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# GROWTH

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# GROWTH

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# CONCOMITANT VARIATION OF MEASURES OF BODY SIZE ON BOYS 5 YEARS OF AGE AND MEASURES OF GROWTH RATE FOR THE ENSUING SEXENNIUM*

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(Received January 2, 1958)

KNOWLEDGE is meager for *Homo sapiens* on the association between size of the organism at a specified age and growth rate of the organism over a subsequent period. The present investigation serves to lessen the paucity of research on this topic.

For each of 15 anatomic traits representing the head, face, trunk and extremities, study is made of covariation in magnitude at age 5 years and change, both absolute increase and percentage gain, during the succeeding sexennium of childhood. The Pearson product-moment formula $r$ is used throughout.

## SUBJECTS AND DATA

The subjects are American-born white boys, predominantly of northwest European lineage and above average socioeconomically. They are characterized more fully in an earlier publication (7).

Measurements of every subject were obtained at two postnatal ages, 5 years and 11 years. The series of records for a particular trait at age 5 years was used in correlation as the X (size) variable. Data for the Y (growth rate[1]) variables were derived as follows: For a given subject and trait,

1. Absolute gain over a six-year period was obtained by subtracting size at age 5 years from size at age 11 years.

2. Relative increment during the same sexennium was obtained by dividing the absolute gain between 5 and 11 years by the size at 5 years and multiplying this quotient by 100.

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[1] For a concise exposition on absolute and relative rates of growth see reference 1, pp. 25-28.

Twelve of the 15 traits were obtained by direct measurement, three were secured roentgenographically. Those obtained through direct measurement were glabello-occipital length of the head, biparietal width of the head, bizygomatic width of the upper face, biacromial width of the shoulders, thoracic circumference at the level of the xiphisternal junction, bi-iliocristal width of the hips, maximum circumference of the left upper limb in the region of the deltoid insertion, stem length from the vertex to the inferior plane of the ischia, lower limb length from the inferior plane of the ischia to the plantar surface of the feet, maximum circumference of the left lower limb in the region of the calf, stature from the vertex to the plantar surface of the feet, and total weight of the nude body. Detailed description of the anthropometric procedures is available elsewhere (10, 13, 6, 3).

The traits secured by roentgenographic methods were bigonial width of the lower face, nose height from the nasion to the anterior nasal spine, and a measure of face depth. Previous reports have described the steps in deriving lower face width (12) and nose height (9). Face depth was derived from *norma lateralis* roentgenograms as the distance from a point on the anterior border of the maxilla 0.8 cm. below the nasal spine to a point representing the anteriormost extension of the occipital condyles.

It is pertinent to state that the measures for each trait were amassed through laboratory practices carefully initiated and maintained to assure high validity (3, 8, 12). For the 12 traits investigated by direct measurement the sample comprises 100 boys enrolled for study between 1940 and 1948; N is 57 for each of the other traits as roentgenograms were obtained only on those boys enrolled after 1945.

## Covariation of Size and Absolute Gain

Table 1 presents the 15 coefficients for size at age 5 years correlated with absolute gain between age 5 years and age 11 years. Inspection of the *r* and P columns of Table 1 shows:

1. In 7 instances the null hypothesis can not be rejected. For length and breadth of head, breadth and depth of face, height of of nose, and breadth of shoulders, there is no manifestation of a statistically significant association between magnitude at age 5 years and absolute increment during the succeeding sexennium.

2. In 6 instances it is reasonable to reject the null hypothesis. One may infer with high confidence that there is positive correlation of size at age 5 years and absolute gain over the ensuing sexennium for circumference of thorax, arm and leg; length of lower limbs; stature; and body weight.

None of the obtained coefficients denotes a strong association; consequently, none provides a highly dependable medium for prediction. Efficiency in prediction[2] is less than 10 per cent for all of the

TABLE 1

Statistics for 15 Traits on North American White Boys Depicting (a) Covariation of Size at Age 5 Years and Absolute Gain during the Ensuing Sexennium, (b) Range of Each X (Size) and Y (Absolute Gain) Distribution.

| Trait | $r$ | P* | $\text{Range}_x$ | $\text{Range}_y$ |
|---|---|---|---|---|
| Head length | —0.09 | .... | 3.1** | 9.0† |
| Head width | 0.05 | .... | 2.7 | 5.7† |
| Upper face width | 0.09 | .... | 2.1 | 11.2† |
| Nose height†† | —0.03 | .... | 1.1 | 5.1† |
| Face depth†† | 0.13 | .... | 1.7 | 11.0† |
| Lower face width†† | 0.17 | .... | 1.3 | 6.0† |
| Stem length | 0.23 | <0.05 | 12.0 | 7.1 |
| Shoulder width | 0.13 | .... | 5.5 | 4.3 |
| Arm girth | 0.41 | <0.01 | 8.7 | 10.1 |
| Thorax girth | 0.36 | <0.01 | 13.3 | 24.5 |
| Hip width | 0.23 | <0.05 | 4.1 | 4.7 |
| Lower limb length | 0.56 | <0.01 | 11.9 | 8.6 |
| Leg girth | 0.35 | <0.01 | 8.2 | 9.7 |
| Stature | 0.42 | <0.01 | 23.6 | 13.3 |
| Body weight | 0.55 | <0.01 | 13.9††† | 36.0††† |

* Probability that the population $r$ is zero (see reference 2, p. 227).
** Each range is given in centimeters unless marked otherwise.
† Range in millimeters.
†† N = 57 for these traits; for all other traits N = 100.
††† Range in kilograms.

traits studied except lower limb length and body weight, and in size-gain predictions for these traits there remains more than 80 per cent of the element of chance. It follows that information on the *size* of an individual boy at age 5 years is of little or no value in estimating the amount of his *absolute change* during the childhood period extending from age 5 years to age 11 years.

Simmons and Todd (14) studied associations between "stature and weight status" at early childhood ages and "the respective increment" during annual intervals following these ages. Their findings from

[2] Derived from the forecasting formula, E (See reference 2, pp. 410-411).

TABLE 2
Correlation Statistics on White Boys for (a) Size at Birth with Absolute Gain during the First Postnatal Year and (b) Size at 1 Year with Absolute Gain over the Ensuing Quadrennium.

| Trait and Source | Birth with First Year | | | 1 Year with 1-5 Years | | |
|---|---|---|---|---|---|---|
| | N | r | P* | N | r | P* |
| Body weight: | | | | | | |
| Simmons & Todd (14) | 88 | —0.13 | .... | | | |
| Low data (4) | 66 | 0.03 | .... | 66 | —0.15 | .... |
| Stature: | | | | | | |
| Simmons & Todd (14) | 64 | —0.46** | <0.01 | | | |
| Low data (4) | 66 | —0.04 | .... | 66 | —0.14 | .... |
| Head length: | | | | | | |
| Low data (4) | 66 | —0.41 | <0.01 | 66 | —0.39 | <0.01 |
| Head width: | | | | | | |
| Low data (4) | 66 | —0.54 | <0.01 | 66 | —0.33 | <0.01 |
| Hip width: | | | | | | |
| Low data (4) | 66 | —0.09 | .... | 66 | —0.59 | <0.01 |
| Foot length: | | | | | | |
| Low data (4) | 66 | —0.19 | .... | 66 | —0.37 | <0.01 |

* Probability that population $r$ is zero.
** This may be an erroneous statistic. The comparable $r$ for girls reported by Simmons & Todd is —0.01 (N = 41).

correlating size at birth with absolute gain during the first postnatal year are reproduced in Table 2. Other findings on boys include $r$'s of 0.08 for stature (N = 129) and 0.14 for weight (N = 132) from correlating size at age 2 years with absolute gain during the third year, and $r$'s of 0.26 for stature (N = 114) and 0.34 for weight (N = 114) from correlating size at age 4 years with absolute gain during the fifth year.

Data covering the age period from birth to 5 years were available from Low (4). Included were records on each of 66 boys for body weight, stature, head length and breadth, bitrochanteric width of hips, and foot length. These data were analyzed by the writers to obtain $r$'s for (a) size at birth with absolute gain during the first postnatal year and (b) size at age 1 year with absolute gain during the period from 1 year to 5 years. The results are displayed in Table 2. It will be seen:

1. There are statistically significant negative associations between head size at birth and absolute change in head size during the first postnatal year, also between head size, hip width and foot length at age 1 year and absolute increase of these dimensions over the period between age 1 year and age 5 years.

2. The null hypothesis can not be rejected for correlation of birth status in body weight, stature, hip width and foot length with absolute increment of these traits over the first postnatal year. Nor is stature or weight at age 1 year associated significantly with the respective gain in stature or weight during the succeeding quadrennium.

## Covariation of Size and Percentage Gain

As emphasized by Minot (11) over 60 years ago, biologic growth is depicted more adequately through "percentage increments" than through "absolute increments." This perspective is illustrated by noting that while two absolute increments of 1.0 kg. are identical mathematically, they are not identical biologically when one is added to an organism weighing 4.0 kg. and the other augments an organism weighing 40.0 kg. Computation of relative increment, or percentage gain, takes account of both absolute gain and "amount of body substance present." All relative increments utilized in the present study, following Minot, were obtained by expressing absolute increase during a specified interval as a percentage of attained size at the beginning of the interval.

Table 3 lists $r$'s for each of 15 traits secured from correlating size at age 5 years with percentage increase over the interval from age 5 years to age 11 years. It is found:

1. All of the coefficients are clustered in the concomitance area extending from low negative, through zero, to low positive. For no trait studied is it possible to predict percentage increment during the sexennium 5 to 11 years, from size at 5 years, with as much as 7 per cent improvement on the mean gain "best guess."

2. The null hypothesis can not be rejected for 9 of the traits. Explicitly, the view that size at age 5 years and relative growth rate during the succeeding sexennium are associated is given no support by the statistics of Table 3 for width of head, depth of face, width of lower face, length of body stem, width of hips, length of lower limbs, circumference of leg, stature, or body weight.

3. In 3 instances the null hypothesis can be rejected at a high level of confidence. Accordingly, it appears tenable to infer that for length of head, width of upper face, and height of nose there is a low negative relationship between magnitude at age 5 years and percentage increase from 5 to 11 years.

Meredith (5), using small samples of infants, investigated the

TABLE 3

Statistics for 15 Traits on North American White Boys Depicting (a) Covariation of Size at Age 5 Years and Percentage Gain during the Ensuing Sexennium, (b) Range of Each X (Size) and Y (Percentage Gain) Distribution.

| Trait | $r$ | P* | $Range_x$ | $Range_y$ |
|---|---|---|---|---|
| Head length | —0.28 | <0.01 | 3.1** | 5.2 |
| Head width | —0.13 | .... | 2.7 | 3.9 |
| Upper face width | —0.27 | <0.01 | 2.1 | 7.8 |
| Nose height† | —0.35 | <0.01 | 1.1 | 12.5 |
| Face depth† | 0.02 | .... | 1.7 | 12.1 |
| Lower face width† | —0.18 | .... | 1.3 | 8.1 |
| Stem length | —0.11 | .... | 12.0 | 11.2 |
| Shoulder width | —0.23 | <0.05 | 5.5 | 16.6 |
| Arm girth | 0.24 | <0.05 | 8.7 | 59.2 |
| Thorax girth | 0.23 | <0.05 | 13.3 | 44.8 |
| Hip width | —0.03 | .... | 4.1 | 26.1 |
| Lower limb length | 0.02 | .... | 11.9 | 16.1 |
| Leg girth | 0.11 | .... | 8.2 | 41.7 |
| Stature | —0.07 | .... | 23.6 | 11.4 |
| Body weight | 0.17 | .... | 13.9 | 138.0 |

* Probability that the population $r$ is zero.

** With one exception, each range in this column is in centimeters; the range for body weight is in kilograms.

† N = 57 for these traits; for all other traits N = 100.

"association between magnitude of stature and percentage gain in stature." The $r$'s reported are —0.36 for stature at birth with percentage gain during the first postnatal year (N = 25, both sexes), and —0.12 for stature at age 1 year with percentage gain during the second postnatal year (N = 39, both sexes). Both coefficients are nonsignificant statistically.

Table 4 presents correlation statistics from analysis of the Low data (4). Principal findings are:

1. There is a moderate inverse relationship between magnitude at birth and relative increase over the first postnatal year. Concomitance is weaker for stature (—0.34) than for body weight (—0.67).

2. There is a moderate inverse relationship between magnitude 1 year after birth and relative increase during the succeeding quadrennium. Again, concomitance is less marked for stature (—0.43) than for body weight (—0.63).

3. Both series of coefficients for head length, head breadth, and foot length fall within the correlation zone —0.4 to —0.7. The forecasting formula, E, shows that in utilization of a best-fit rectilinear regression equation, where $r$ = —0.7 error of prediction is reduced by 29 per cent.

TABLE 4
Correlation Statistics on White Boys* for (a) Size at Birth with Percentage Gain during the First Postnatal Year and (b) Size at 1 Year with Percentage Gain over the Ensuing Quadrennium.

| Trait | Birth with First Year | | 1 Year with 1-5 Years | |
|---|---|---|---|---|
| | $r$ | P** | $r$ | P** |
| Body weight | —0.67 | <0.01 | —0.63 | <0.01 |
| Stature | —0.34 | <0.01 | —0.43 | <0.01 |
| Head length | —0.58 | <0.01 | —0.50 | <0.01 |
| Head width | —0.70 | <0.01 | —0.42 | <0.01 |
| Hip width | —0.31 | <0.05 | —0.73 | <0.01 |
| Foot length | —0.43 | <0.01 | —0.62 | <0.01 |

* Calculated from data accumulated by Low (4); N = 66.
** Probability that population $r$ is zero.

## SUMMARY

This investigation contributes to knowledge on the association between size of the organism at a specified age and growth rate of the organism over a subsequent period. The subjects are white boys. Primary attention is focused on 15 traits studied with reference to the covariation of magnitude at age 5 years and increment during the ensuing sexennium. Supplementary study is made for 6 traits relating size at birth to increase during the first postnatal year, and size at age 1 year to change over the succeeding quadrennium.

Correlation findings are reported for (1) size with absolute gain, and (2) size with percentage gain. The latter are considered to have the greater biologic import. Major generalizations are as follows: (1) there is a moderate negative association between body size at birth and percentage gain during the first year of infancy, (2) there is a moderate negative association between magnitude 1 year after birth and percentage gain over the succeeding quadrennium of early childhood, and (3) there is concomitance varying from low inverse, through zero, to low direct for size at age 5 years and percentage increase during the ensuing sexennium of late childhood. Obviously each generalization has limited scope, applying in essence to the particular series of anatomic traits investigated.

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# THE EARLY TURKEY EMBRYO, ON MEDIA PREPARED FROM EGGS OF "HIGH" AND "LOW" HATCHABILITY HENS[1,2]

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(Received October 7, 1957)

THE problem of hatchability in the domestic fowl is a very real one to the practical poultry breeder and hatcheryman. To the student of growth and differentiation, who attempts to examine the underlying mechanisms responsible for variation in hatching capacity, it can be an elusive and even baffling one, indeed. Its inherent difficulty is due, principally, to the fact that hatchability, in part, is a zygotic characteristic and, in part, is controlled by extra-embryonic "maternal" factors. Furthermore, the genetic capacity to hatch can be greatly modified by environmental factors and may be different for each flock at different times.

A definite relation between early embryonic development and hatchability has been shown by Bronkhorst (1933), Olsen and Marsden (1954), and in this laboratory (unpublished). There is a retardation of growth and a relatively higher peak of mortality for "low" hatching embryos than for "high" hatching embryos during the first week of growth. The causes of this early retardation and mortality may lie in the genetic constitution of the embryo, or in its immediate environment.

The genetic factors affecting hatchability have been discussed in detail by Landauer (1951). Wilson and Johnson (1946) reported a heritability ($h^2$) of hatchability (i.e., that fraction of the total variation associated with hatchability which is accounted for by heredity) of 0.26 for turkey eggs, while in the study of Abplanalp and Kosin (1953) the estimates of $h^2$ ranged between 0.03 and 0.21. According

[1] Scientific paper No. 1661, Washington Agricultural Experiment Stations, Pullman. Projects Nos. 803 and 1225.

[2] Supported, in part, by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and by Federal Funds for Regional Research (W-7) under the Hatch Amended Act.

to Shoffner and Sloan (1948), the corresponding value in chickens is 0.16.

The effects of maternal factors on hatchability are also well recognized. Abplanalp and Kosin (1953) reported this influence to be of the order of 0.05. Brunson, et al. (1956), and Crittenden, et al. (1957) obtained estimates of 0.13 and 0.039, respectively, for chickens. These findings suggest that "the dams exhibited an apparently greater influence on the performance of the eggs they laid than the sires with which they mated" (Crittenden, et al., 1957).

However, whether the physical structure of the shell, the chemical composition of the yolk and albumen, or the genetic contributions from the dam are involved in the capacity of the egg to hatch cannot be determined from these estimates. For example, Munro (1940) has reported a relation in chicken eggs between specific gravity of the egg and its hatching power. The existence of a relation between egg weight, shell color and hatchability has been reported for chickens (Scott and Warren, 1941 and Godfrey, 1947) and for egg weight in turkeys (Insko, et al., 1943). In turkeys, the shape of the egg may also be a factor in hatchability (Kosin, 1957).

Information on the relation between nutrition and hatchability is plentiful. Romanoff (1948) stated succinctly: "It is not a new discovery that the diet of the hen affects the composition of the egg, and that nutritional content of the egg in turn profoundly influences the course of embryonic development. It is well known that deficiencies in the egg may make it impossible for the embryo to live long enough to hatch." However, whether or not "nutritional" deficiencies exist in the yolk and albumen of eggs from a genetically isolated line of low hatching hens has not been demonstrated. The *in vitro* culture technique described by Spratt (1947a), whereby the embryo may be cultivated in the near or complete absence of its own yolk and albumen, permits an examination of this question. Thus the importance and extent of influence of these extra-embryonic maternal factors can be evaluated by comparing the growth and differentiation of embryos explanted on media prepared from the yolk and albumen of eggs from either high or low hatchability hens.

## Materials and Methods

The eggs used in this study were obtained from 39 Broad Breasted Bronze turkeys hatched between April 13 and April 27, 1956. The

first eggs were obtained on January 11, 1957. The hens were trapnested and the eggs collected three times a day. The eggs were held at room temperature for a short period, usually overnight, and then stored at 53° F. and 85% relative humidity. All eggs were incubated within three days of being laid. After 36 to 40 hours of incubation, a majority of the embryos were in the head process or head fold stage (stage 5 and 6 of Hamburger and Hamilton, 1951). However, all embryos from the definitive primitive streak stage (stage 4) to embryos with 6 somites (stage 8) were explanted following the technique outlined by Spratt (1947a). The 36 to 40 hour turkey embryo was chosen as the stage to be explanted because of its still relatively simple morphology.

The "high hatchability" medium was prepared (Spratt, 1947b) from the yolk and albumen of eggs from a selected group of 9 hens which had an average per cent fertile hatch of 91.7. The "low hatchability" medium was prepared from a selected group of hens with an average per cent fertile hatch of 72.6. The difference in hatchability between these two groups was statistically significant. It should be pointed out that in a majority of cases, the two media actually were prepared from eggs of hens with greater than 50% difference in hatchability.

To make the nutrient conditions approach as closely as possible those found in the developing egg, only fertile eggs were used to prepare the media. The albumen was first separated from the yolk of one or more eggs which had been incubated for 36 to 40 hours. The embryo was removed by cutting around the blastoderm with scissors and the yolk was permitted to flow out of the opened yolk sac. The embryo and the yolk sac were discarded. The yolk and albumen mixture was shaken vigorously for 30 seconds and an equal volume of 0.123 M sterile saline (Howard, 1953) was added and thoroughly mixed. After settling for 15 to 30 minutes, the liquid portion was centrifuged for 45 to 60 minutes at 2200 to 2400 RCF. The supernatant was poured off and mixed with an equal amount of saline-agar (0.7 gm. % Special Noble agar) kept at 42° C. The yolk-albumen-agar-mixture was then poured on watch glasses set on moistened cotton rings in Petri plates and permitted to harden. Pyrex doubly distilled water was used in the preparation of all media.

The explanted embryos were periodically observed from 6 to 48 hours after explantation at intervals of 8 to 10 hours. The devel-

opment of the following organs was recorded: somites, brain, neural tube, optic vesicles, otocysts, heart and brain vesicles. The embryos were "staged" at 24 to 26 hours according to the stages of normal development established for the chick embryo by Hamburger and Hamilton (1951). The length of the embryo from the tip of the head to Hensen's node and the anterior-posterior length of the area pellucida were measured with an ocular micrometer. All abnormalities were sketched or photographed, and recorded.

## Results and Discussion

The stage of development of the embryo at explantation was highly correlated with the length of the embryo and the anterior-posterior length of the area pellucida (Table 1). Thus, any one of these

TABLE 1
Correlation between stage of development and length measurements.

| Variables | r |
|---|---|
| Somite number and embryo length | 0.91 |
| Stage and embryo length | 0.95 |
| Ectodermal derivatives and embryo length | 0.93 |
| Somite number and area pellucida length | 0.77 |
| Stage and area pellucida length | 0.85 |
| Ectodermal derivatives and area pellucida length | 0.83 |

criteria-stage of development, somite number, embryo length, or length of the area pellucida—can be used in describing the growth of the embryo *in vitro*. The high correlation between somite number and embryo length (0.91) completely agrees with the findings of Fischel (1896) for duck embryos. An analysis of his data by Bernier et al. (1951) shows the correlation coefficient of 0.91 between the two variables.

An analysis of the covariance of final lengths adjusted for initial lengths of all embryos, whether explanted on medium prepared from high or low hatchability eggs, showed no significant difference between treatments or among hens (Table 2). Thus the type of medium, as used in this study, had no effect on the early development of turkey embryos. Both media were entirely sufficient for maintaining the development of the embryo, *in vitro,* for 24 to 27 hours after explantation. This is not to say, however, that the two media are necessarily identical. There may be differences in the vitamin, enzyme, or hormone content in the yolk and albumen of the two kinds

TABLE 2

The effects of treatment on the *in vitro* development of the 36 to 40 hour turkey embryo according to the stage at explantation.

| Stage at Explantation. | | 4 | | 5 | | 6 | | 7 | | 8 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Media | | High | Low | High | Low | High | Low | High | Low | High | Low | |
| | 4 | 5(10)* | | | | | | | | | | |
| | 5 | 1(2) | | | | | | | | | | |
| Stage of de- | 6 | 1(2) | | 1(1) | | | | | | | | |
| velopment | 7 | 8(15) | 14(27) | 1(1) | | 1(1) | | | | | | |
| 24 hours | 8 | 21(40) | 21(41) | 20(29) | 21(29) | 2(2) | 2(2) | | | | | |
| after ex- | 9 | 13(25) | 16(31) | 34(49) | 41(56) | 23(19) | 26(23) | 1(2) | | | | |
| plantation | 10 | 3(6) | | 13(19) | 10(14) | 57(47) | 41(36) | 1(2) | 3(5) | | 1(4) | |
| | 11 | | | 1(1) | 1(1) | 39(32) | 42(37) | 10(22) | 15(26) | | | |
| | 12 | | | | | | 2(2) | 33(73) | 39(68) | 6(22) | 4(16) | |
| | 13 | | | | | | | | | 21(78) | 20(80) | |
| | 14 | | | | | | | | | | | |
| Total | | 52 | 51 | 70 | 73 | 122 | 113 | 45 | 57 | 27 | 25 | 635 |

* Figures in parentheses give distribution of explanted embryos in percentages.

of eggs that went into the making of these media. However, these differences do not appear to be critical in eggs with greater than 19% difference in hatchability.

To demonstrate the necessity of some substrate besides agar for the normal development of the turkey embryo *in vitro*, more than 30 embryos were explanted on a plain saline-agar (0.4 gms. %) medium. The embryos disintegrated within 12 hours, though in a few cases large thin vesicles remained. This indicates that additional nutrient material besides that provided by the medium was necessary to sustain development. The amount of nutrients, carried over in the process of explantation as well as that present in the embryo itself, was not sufficient to sustain the early growth and differentiation of the embryo. It appears that causes of this early retardation of growth and mortality of low developmental potential embryos probably lie elsewhere than in its yolk and albumen. The question of whether or not high and low developmental potential embryos differ in their growth patterns *in vitro* is now under investigation.

## Summary

The possible effects of extra-embryonic maternal factors on the development of the turkey embryo were examined by comparing the growth and differentiation of the 36 to 40 hour turkey embryo on a medium prepared from the yolk and albumen of high or low hatchability eggs. The eggs were obtained from two groups of hens with an average difference in hatchability of greater than 19%. In a majority of cases, eggs from hens with greater than 50% difference in hatchability were used. The medium prepared from the eggs of low hatchability hens was found to be sufficient for sustaining the normal growth and differentiation of the turkey embryo, *in vitro*, for 24 to 27 hours. An analysis of the covariance of final lengths on initial length of all embryos showed no significant difference between treatments or among hens. The length of the embryo, somite number, length of the area pellucida, and the stage of development were highly correlated variables.

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# A STUDY OF COLCHICINE INDUCED STERILITY IN THE FEMALE FRUIT FLY *DROSOPHILA MELANOGASTER*

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(Received March 24, 1958)

## INTRODUCTION

CHEMICAL induction of sterility, unlike that produced by ionising radiations, is relatively unexplored in insects. Gelei and Csik (1940) found that when *Drosophila* were fed with certain proportions of colchicine they died after a couple of days without offspring. Hadorn (1946) treated larval ovaries in vitro with varying concentrations of colchicine solution, transplanted them to normal larvae and observed the injurious effects produced. Fabian (1947) investigated the effects of different concentrations of injected colchicine on adult female *Drosophila* and observed that they were rendered completely or temporarily sterile. Goldsmith and Frank (1952) induced sterility in *Drosophila melanogaster* by feeding a folic acid antagonist and Mitlin et al. (1957) observed that the feeding of house flies on mitotic poisons prevented ovarian development and caused sterility in the female. In these instances the resulting sterility in the adults was mainly explained on such criteria as the absence of egg laying, delayed oviposition, reduction in the number of progeny, etc. The present study deals with the sterility induced by feeding colchicine and also the morphological and cytological changes that lead to the sterility.

## MATERIAL AND METHODS

By conducting a series of experiments using various amounts of colchicine, the suitable amount of this chemical, which when mixed with the usual *Drosophila* medium was tolerated by the flies without visible ill effects, was determined. It was found that in 0.0025% colchicine medium the flies could be maintained for about six days without severe ill effects. After this period they developed what may be called colchicine-oedema, manifesting a bloated abdomen and mouth parts owing to heavy accumulation of fluid in the haemocoele.

Accurately timed eggs of Ore-K, wild type strain of *Drosophila melanogaster* inbred in this laboratory were collected by the usual method. They were then transferred to the normal medium, the resulting larvae sexed at the appropriate time and males and females allowed to develop separately. Just eclosed virgin females were transferred to the experimental medium where they remained for varying periods. Flies in normal conditions were also maintained to serve as controls for comparison.

The ovaries were dissected out as and when required, fixed in 1:3 acetic alcohol, stained in Feulgen, and whole mounts of ovaries and ovarioles made. The terminology of King et al. (1956) who described the oogenesis in normal flies have been followed in this study.

## Results

In the first series of experiments the flies were treated for three days. It is known that normal females begin to lay eggs on the third day after eclosion, the rate of oviposition reaching a maximum on the fourth day, and hence the inhibiting effects of colchicine could be readily noted during this period. At the end of the three day treatment it was seen that the size of the ovary was markedly smaller compared to normal ovary of the same age (Fig. 4). The ovaries of treated females showed individual variation in size but in about 60% of the flies they were more or less of the same size and this (Fig. 5) was considered to represent the three day treated ovary. In the remaining flies the ovaries were of the size of just eclosed females (Fig. 1) or smaller. In these cases there has therefore been total inhibition of ovarian development, and in those represented as characteristics of three day treated it may be noted that the presence of 2-3 small eggs in each ovary is the only advance over the just eclosed condition.

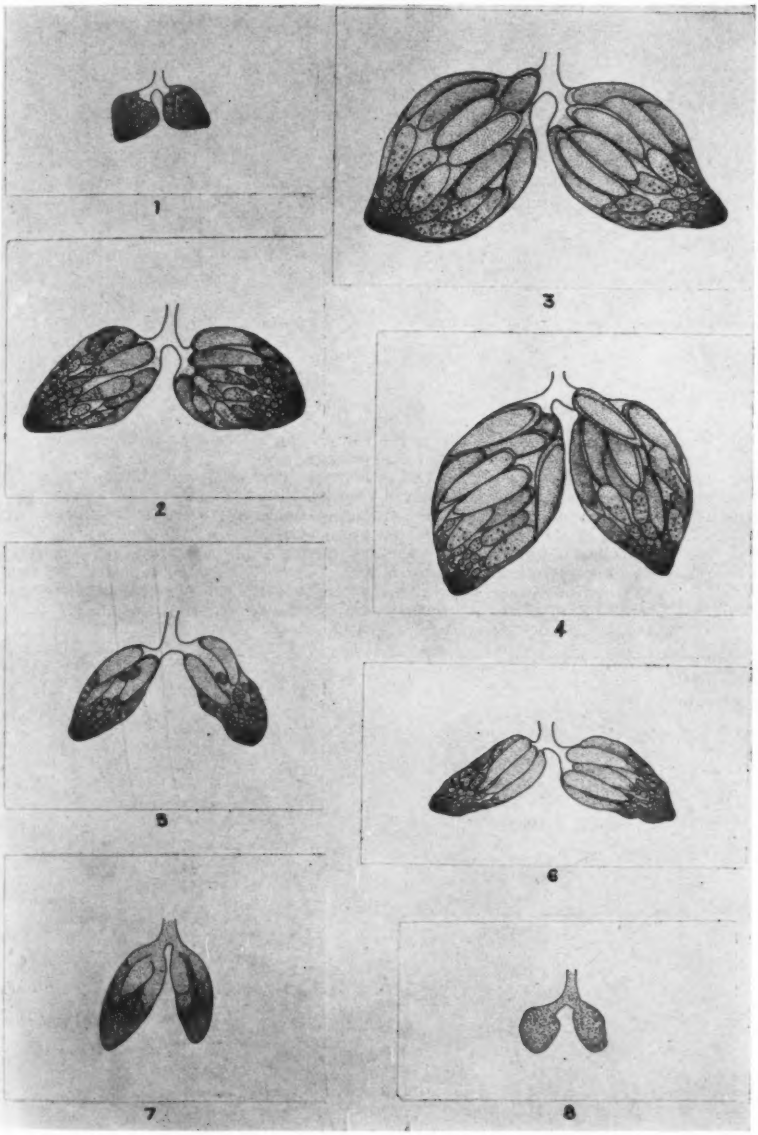
The study of the individual ovarioles showed that the germaria were severely affected. In some cases the tip of the germarium was breaking up, while in its posterior region there were present cells much bigger than in the normal case, and some of these big cells showed numerous chromatin elements, Fig. 9. These cells may be polyploid and showed no tendency to be organized into a cyst as would normally be the case in stage 1. In other ovarioles the germaria were reduced to a slight bulge on stage 3 chamber, Fig. 10. Stages 1 and 2 were seldom seen. Chambers 3, 4, 5, 6 and 7 were common but their

nurse nuclei stained very faintly in Feulgen, and especially in the later stages they seemed to be highly deficient in chromatin material. In stage 7 the nurse nuclei showed very faintly stained and granular chromatin amidst which were many Feulgen-negative spheres as shown in Fig. 11. In such chambers the oocyte nucleus was about the size of the nurse nuclei and appeared empty except for a nucleolus which also seemed to be Feulgen-negative. In some ovarioles a later stage which may be referred to as stage 8 was found. This was not much bigger in size than stage 7 described above, but the oocyte appeared to be separated from the nurse cells as a protrusion from the main body of the chamber, Fig. 12. The nurse nuclei were larger than before but presented much the same appearance. The abnormal stage 7 chamber found will be described later. The next and final stage in the ovary was the eggs, much smaller than normal ones and some probably were in stage 12 . The dorsal appendages were absent or highly reduced.

In four day treated flies there was also variation in the size of the ovaries, but they could be grouped in two classes as in the three day treated flies. In 20-30% of the treated fiies the size of the ovary remained about that of the three day treated, but in the remaining larger percentage a slight increase was noted with 4-5 eggs in each ovary (Fig. 6). Some of these eggs were in stages 12 of 13, showing remnants of nurse nuclei in the anterior regions. Eggs with nurse nuclei scattered all over the yolk were also sometimes seen. The dorsal appendages were absent or rudimentary in the eggs. Since no eggs were laid during the four day period of treatment, and since each of the ovaries contined only 1-2 more eggs in addition to that formed during the three day period of treatment, it is clear that there has been very little developmental activity during the fourth day of treatment. In the ovarioles the germaria were in most cases completely degenerated, but stages 3, 4, 5 and 6 were still seen with little or no chromatin in the nurse nuclei. Stages 7 and 8 as described earlier were found and the Feulgen-negative spheres in their nurse nuclei appeared still more prominent. Abnormal stage 7 chambers as shown in Fig. 13 could be seen in three and four day treated ovaries. The chamber was more elongated and spindle shaped and contained varying numbers of nurse nuclei and heterogeneous masses of chromatin lying scattered all over. In extreme cases the chamber contained only such chromatin masses. The follicular epithelium had broken

## PLATE I

Fig. 1. Ovaries of a just eclosed fly.
Fig. 2. Ovaries of a one day old normal fly.
Fig. 3. Ovaries of a two day old normal fly.
Fig. 4. Ovaries of a three day old normal fly.
Fig. 5. Ovaries of a three day old treated fly.
Fig. 6. Ovaries of a four day old treated fly.
Fig. 7. Ovaries of a five day treated fly.
Fig. 8. Ovaries of a seven day old treated fly.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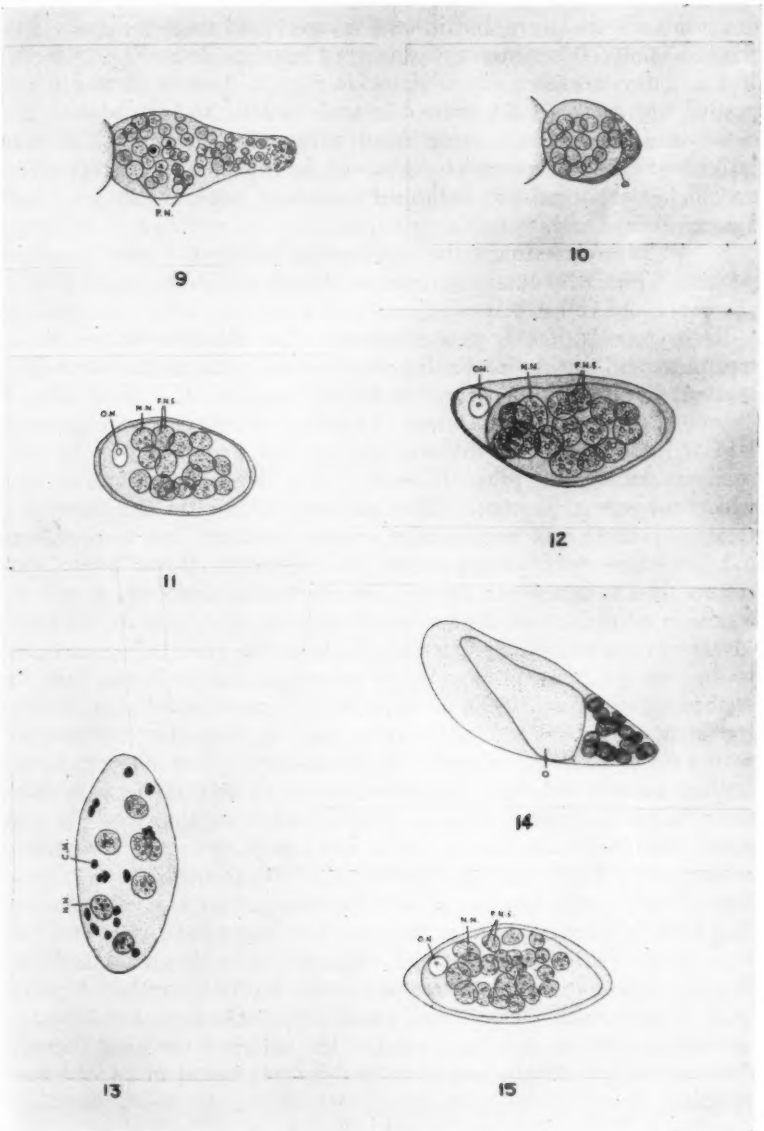
## PLATE II

Fig. 9. A drawing showing the breaking up of the tip of the germarium and the disorganized polyploid nuclei in the posterior region.
Fig. 10. A drawing showing the reduced germarium immediately preceding a stage 3.
Fig. 11. A drawing of an inhibited stage 7, showing the appearance of the nurse nuclei.
Fig. 12. A drawing of a stage 8 formed during treatment, showing the protruding oocyte and the appearance of the nurse nuclei.
Fig. 13. A drawing of an abnormal stage 7 occurring under treatment, showing nurse nuclei and scattered chromatin masses.
Fig. 14. A drawing of a stage 11 occurring during recovery showing attenuated oocyte.
Fig. 15. A drawing of a stage 7 occurring rarely during recovery showing 22 nurse nuclei and 1 oocyte nucleus.


P.N.
9
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O.N.
N.N.
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C.M.
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N.N.
P.N.S.
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down in such chambers, but in other stage 7 and stage 8 chambers it showed faint cell outlines and was very inconspicuous.

A five day treated ovary is shown in Fig. 7. Two or three disintegrating eggs were usually present in such ovaries, and the base of the ovary and the oviducts were filled with yolky material from the broken up eggs. The anterior regions of the ovarioles had disappeared and highly abnormal and deformed chambers, probably stages 7 and 8, were seen.

By seven day treatment the ovaries were reduced to small bag-like pouches (Fig. 8) containing masses of yolky matter, disintegrated chambers and cell debris.

Recovery experiments were conducted after the different periods of treatment and certain interesting observations were made. Three day treated flies were transferred to normal medium and their ovaries examined after six days. During this period of recovery no eggs were laid and the ovaries in the majority of flies had increased in size, each containing 7-8 eggs. However, in a small proportion of flies which correspond to those with smaller ovaries after the three day treatment, there was no indication of any recovery. In the recovering ovaries stages 8-11 were present; their nurse nuclei although well stained in Feulgen never showed the characteristic condition of such stages in normal oogenesis described by King et al. (1956). In stage 11 the oocyte was highly attenuated due to the yolk being restricted to an area as shown in Fig. 14. A somewhat similar oocyte was described by Beatty (1949) in the ovary of the homozygous female-sterile mutant Ocelliless. Abnormal stage 7 and stage 8 chambers with varying numbers of nurse nuclei and chromatin masses, as described before, were present. Rare instances of stage 7 with more than the usual number of nurse cells (up to 22 as shown in Fig. 15) were found. In his study of the irradiated ovary of *Drosophila melanogaster* King (1957b) observed such chambers with excess numbers of nurse cells. In the present instance only one oocyte nucleus could be detected; it was at the posterior end of the chamber, and it could not be said with certainty how such chamber was produced. Beatty (1949) described abnormal chambers with more than 15 nurse cells in the ovaries of the mutant comb-gap, stocks c g c and b pr c g, which have the cg gene in common. He favoured the view that the "super-nucleate follicles" were formed by the fusion of two or more follicles.

In the ovaries of flies treated for four days and then allowed to recover for seven days, stage 10 practically formed the youngest stage in the ovary. About 8-10 small eggs of varying sizes, some of which were very shrunken, were present. Some were more or less round and showed persisting nurse nuclei in the yolk. None of them had the typical appendages. Even in the ovaries recovering after three day treatment the anterior regions of the ovarioles beyond stage 7 had disappeared and this indicates that the earlier stages were damaged beyond recovery by the treatment.

Five day treated ovaries did not recover at all. No ovarioles could be seen after a five day period of recovery and the ovaries were reduced to short tubular structures containing yolky material, disintegrated chambers and nuclei. It was evident from the size and morphology of the seven day treated ovaries that there was no question of any recovery in them.

## Discussion and Conclusions

In normal conditions the increase in size of the ovary from eclosion to the end of the first day is tremendous, as may be seen from Figs. 1 and 2. The growth during the next day is no less remarkable, and by the end of two days after eclosion (Fig. 3) the ovary attains about its maximum size during the whole adult life. From the observations of colchicine fed flies it is clear that a three day treatment from eclosion severely inhibits ovarian growth, completely prevents oviposition and thus effectively sterilises the flies. The most noticeable effect in the treated ovaries was the destruction of the germaria, as explained earlier. The course of events leading to this was probably as follows. First, the complete cessation of mitotic activity, as no cysts appeared to have formed after treatment began; secondly, polyploidization of the existing cells, evidence for which was the large size of the cells; and finally the breaking up of such cells. The rarity of stages 1 and 2 was already mentioned. Excepting these observations, and the occurrence of the 2-3 small eggs, the distribution of the existing stages in the three day treated ovary was more or less similar to that in the just eclosed ovary. However the nurse nuclei in the different stages present showed very faintly stained nuclei, and those in chambers up to and including stage 6 appeared gradually to contain less and less chromatin and were probably killed by the end of four days treatment. Recovery experiments showed their inability

to revive, and indicated that they were irrevocably damaged even by three day treatment. But for the eggs, stage 7 mainly formed the final stage in the three and four day treated ovaries, and since this stage was found in substantial numbers in just eclosed ovaries, they must have persisted all along during the period of treatment. But they have been generally inhibited from overall growth. Most of them remained stationary in size, and though as a whole appeared normal, their nurse nuclei were very faintly stained in Feulgen and showed granular chromatin and many Feulgen-negative spheres. A few stage 7 chambers were abnormal as explained before, and some others must have continued to develop very slowly under the treatment to account for the stage 8 and the small eggs formed by the end of three days. Under continued treatment some more stage 7 seemed to have developed, as shown by the occurrence of 1-2 more eggs in four day treated ovaries. Further stages 8-11 and more number of eggs were seen in ovaries recovering after three and four day treatments; these must have developed from the inhibited stage 7. All these facts suggest the conclusion that the treatments mentioned did not seriously damage most of the stage 7 chambers. But it was seen that treatment beyond four days resulted in such damage from which there was no recovery.

From the varying susceptibility of the different stages to treatment, we may consider oogenesis to consist of three phases of which the germarium, with its mitotically active cells that are most easily affected, is the first. Later stages, including stage 6, in which further development is completely stopped and which gradually succumb, constitute the second phase. Stage 7, whose behaviour has been explained above, and the later stages form the third phase. Now it is a known but unexplained fact that in normal flies mature eggs are not produced in substantial quantities for at least 48 hours, although freshly hatched females contain substantial numbers of stage 7 oocytes (King 1957a). Therefore it seems that stage 7 is a very stable one, and that at this stage there is a natural pause in development. These phenomena are exemplified under the experimental conditions.

During treatment, stage 7 chambers generally did not increase in overall size, perhaps because of lack of yolk formation and growth of the oocyte. In their nurse nuclei, which also had not increased in size, the occurrence of the Feulgen-negative spheres was mentioned

before. King et al. (1956) described such spheres or plasmosomes as filling the nurse cell nuclei of normal stage 10. In the present instance the occurrence of such spheres in stage 7 may be interpreted as an indication of some continuing activity of the nuclei. In this connection it must also be pointed out that these nuclei when compared to the nurse nuclei in normal stage 7 were highly deficient in chromatin and there may be some connection between this and the lack of yolk formation.

In his study of the irradiated ovary of *Drosophila melanogaster* King (1957b) observed chambers which could be classified as stage 8 on the basis of overall size, follicular morphology, the size of the oocyte and nurse nuclei, but the nurse nuclei were morphologically identical with those of stage 3 or 4. This means, as he stated, an arrest of nuclear differentiation in the nurse cells, but not growth of these nuclei, their cytoplasm and above all yolk synthesis or the differentiation of the follicular epithelium. The study of stage 7 and later stages in colchicine treated ovaries and also in recovering ones suggest different conclusions. The stage 7 chambers were generally inhibited from overall increase in size, with no formation of yolk and growth of the oocyte, but their nurse nuclei appeared to have continued activity to show some differentiation, normally seen in a later stage. Yolk deficiency was seen in later stages and especially in stage 11 of recovering ovaries. Further, as the tubular projections which later become the dorsal appendages are in the first instance produced by the follicle cells and subsequently strengthened by chorionic material (King et al. 1956), the absence of such structures, or the presence of mere rudiments in eggs formed during colchicine treatment, may be attributed to lack of activity of the follicle cells. Fabian (1947) also reported that as a consequence of injection of a certain concernation of colchicine the two horns so characteristic of normal eggs disappeared entirely. It would thus appear that the nature of functional disturbances which make normal development impossible in irradiated and chemically treated ovaries is not the same. Further studies on induced abnormalities in the *Drosophila* ovary may help to give an insight into the mode and time of action of the genes concerned with the abnormalities in the ovary of female-sterile mutants.

## Summary

1. Freshly emerged females treated for three days with 0.0025% colchicine food suffered a severe inhibition of ovarian growth, which completely prevented egg production and caused sterility.

2. The germarium was the first region to be affected and soon broke up, whereas the later stages, including stage 6, were inhibited from further growth and gradually died by the fourth day of treatment. Recovery experiments showed that they did not recover even after a three day treatment.

3. Most of the stage 7 chambers remained alive up to the end of four day treatment, although a few became abnormal, and certain others developed very slowly to form later stages and small eggs by the end of three and four day treatments.

4. The nurse nuclei of the inhibited stage 7 chambers showed some resemblance to those of normal stage 10, in the presence of the Feulgen-negative spheres; the possible significance of this has been discussed. The deficiency of chromatin in the nurse nuclei, the absence of yolk formation and growth of the oocyte and the inconspicuousness of the follicular epithelium have also been pointed out.

5. The stage 8 seen during treatment, and stage 11 in the recovering ovary, were deficient in yolk content. The "eggs" produced under treatment were much smaller than normal ones and lacked the appendages so characteristic of normal eggs.

6. Based on the susceptibility of the different stages to treatment and their subsequent behaviour during recovery, it would appear that oogenesis consists of three phases.

7. It was pointed out how the functional disturbances caused by the colchicine treatment differ from that produced in the irradiated ovary studied by an earlier author.



## Acknowledgements

I am indebted to Professor C. H. Waddington, Sc. D., F.R.S., for his interest in this work and for reading the manuscript. My thanks are due to the technical staff of the Institute for various help during the course of this study. Grateful acknowledgement is also made to the Royal Commission for the Exhibition of 1851, London, for the award of a Scholarship.

## Description of Figures

All figures are camera lucida drawings and semi-diagrammatic. Figures 1-8 are magnified to x ca 20 and the rest to x ca 200.

C.M., chromatin mass; F.N.S., feulgen-negative spheres; G., germarium; N.N., nurse nucleus; O., oocyte; O.N., oocyte-nucleus; P.N., polyploid nuclei.

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# HISTOLOGY OF THE THYROID GLAND OF THE WHITE RAT FOLLOWING INJECTION OF PROGESTERONE

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Our recent study (1) of 65 autopsy thyroids from pregnant women revealed that during pregnancy the thyroid has abundant colloid and at the same time a hypertrophic epithelium. Determination of protein bound iodine (1) in the serum of pregnant women showed a trend toward high values, indicating an increased thyroid activity.

Some writers have suggested that female sex hormones may be responsible for this alteration of the thyroid during pregnancy. This paper is based on the study of the thyroid of the white rat following injection of Premarin and Progesterone.

## Material and Methods

Ninety female virgin white rats of the Sprague-Dawley strain were divided into four groups which were kept in separate metal cages under utmost cleanliness. The basic diet of all animals consisted of Purina Fox Chow and tap water. Thirty-eight white rats served as controls. Thirty animals received daily subcutaneous injections of 1 mg of Progesterone for 7 to 20 days. Ten received, in addition, 2 mg Premarin intraperitoneally, and 10 other animals were injected only with Premarin in the same dosage.

Eighty-six animals survived the experimental period of 7 to 20 days and were killed by exsanguination in Nembutal anesthesia. Paraffin sections of the neck organs, 6 to 8 microns thick, were stained with hematoxylin and eosin.

The diameter of the thyroid follicles and the height of the follicle wall were measured in the central portions of each lobe with the Zeiss micrometer eyepiece.

## Results

Table 1 summarizes the results of our experiments. In the controls, the average diameter of the thyroid follicles was 57 microns

TABLE 1

| Group | No. of Animals | Diet | Hormone | Dosage | Duration | Micron Average Follicle Size | Micron Average Height of Cells |
|---|---|---|---|---|---|---|---|
| 1 | 40 | Purina Fox Chow Tapwater | | | 7-20 days | 57.1 | 7.6 |
| 2 | 30 | Purina Fox Chow Tapwater | Progesterone (Abbott) | 1 mg daily subcutan. | 7 days | 86 | 8.2 |
| 3 | 8 | Purina Fox Chow Tapwater | Progesterone<br>Premarin (Ayerst) | 1 mg daily<br>2 mg intra-peritoneal | 7 days<br>7 days | 48.1 | 8.3 |
| 4 | 8 | Purina Fox Chow Tapwater | Premarin (Ayerst) | 2 mg intra-peritoneal | 7 days | 51.2 | 7.5 |

and the average height of the follicle cells was 7.6 microns (Fig. 1).

There was a striking change in the microscopic picture of the thyroids after injection of Progesterone (Fig. 2). The gland had much more colloid, its consistency was thin and there were many secretory vacuoles. The diameter of the follicles varied from 72 to 98 microns, its average being 86 microns. The epithelial cells lining the follicle lumen were higher than normal, varying from 8.1 to 8.6 microns (Fig. 3).

Sixteen animals which received Premarin either alone or in combination with Progesterone, did not show these histologic alterations. The average follicle diameter in these thyroids measured less than 50 microns and its colloid was much thicker.

## Discussion

Following injection of Progesterone, the thyroids of white rats markedly resembled thyroids as observed in human and animal pregnancy (Fig. 4). They had the same abundance of colloid and the same hypertrophy of thyroid cells. Administration of Premarin did not produce these changes.

Our experiments confirm the observations of Knaus (2) 1923, and Carloni (3) 1939, who reported increase in colloid following injection of corpus luteum extract.

We are not able to say whether Progesterone acts directly on the thyroid cells or by mediation of the hypophysis. The abundance of colloid makes it unlikely that increased pituitary function is responsible for these changes in the thyroid. Thyroid stimulating hormone increases the resorption of colloid and does not stimulate colloid storage.

## Summary and Conclusions

Female virgin white rats were injected daily with 1 mg of Progesterone subcutaneously. After 7 days their thyroids showed the same histologic characteristics as pregnancy thyroids, i.e., abundance of colloid and at the same time hypertrophy of the thyroid epithelium.

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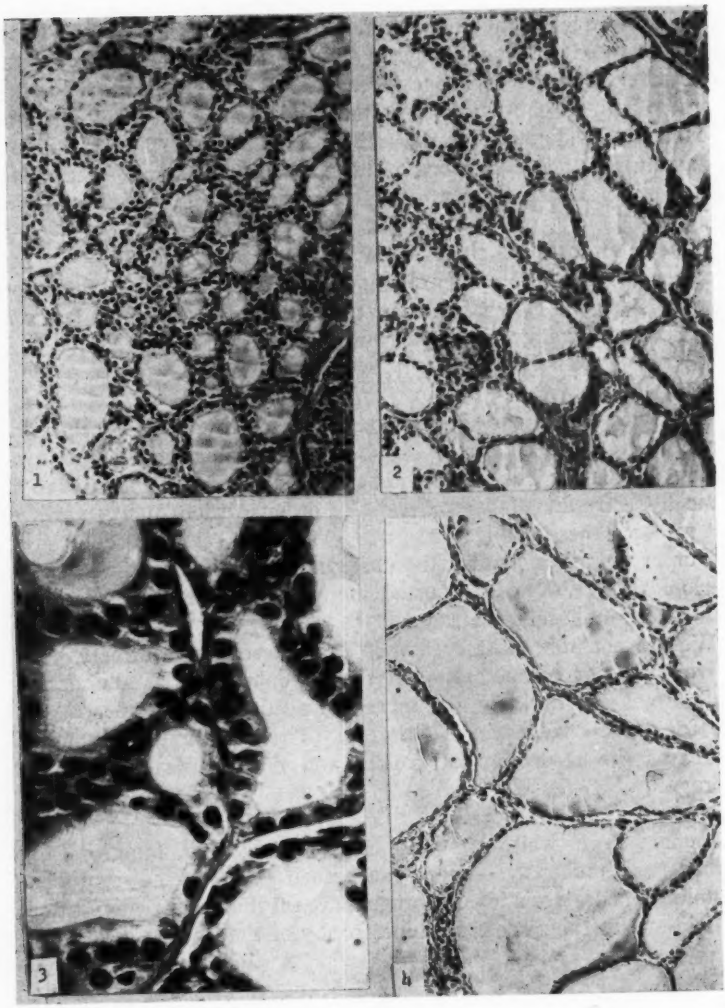


LEGENDS

Fig. 1. Thyroid gland of control animal (Group 1), x 160. Cuboid epithelium, small follicles.

Fig. 2. Thyroid gland of white rats from Group 2, x 160. Progesterone, 1 mg daily for 7 days. Large follicles with abundant thin colloid. High cuboid epithelium.

Fig. 3. High power (x 480) of same thyroid. The high epithelium and the large size of follicles is evident.

Fig. 4. Thyroid of 34 year old pregnant woman in last trimester. The weight was 21 grams and the average diameter of the follicles was 315 microns. The epithelium varied from 2.9 to 11.6 microns.

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# MORPHOLOGY OF THE BLOOD CELLS OF SOME CRUSTACEA

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## INTRODUCTION

THERE are many discrepancies in the literature concerning the morphological characteristics of formed elements of the blood in a number of invertebrates. A great deal of the variation that exists in the classification of blood cells of invertebrates is due to the fragility of these formed elements. Some of the usual techniques such as smear and tissue preparations have not proven applicable in the study of invertebrate blood cells because these cells disrupt readily or become grossly misshapen. Examination of the fresh blood of invertebrates under controlled conditions appears to be a better procedure, but such studies with the use of the light microscope are limited by the optical properties of the cells. The cells are too transparent and therefore only the barest outlines and the more prominent features are recognized in such examinations. It is, therefore, extremely difficult to obtain a clear picture of blood cell morphology by the usual methods of fixation, and staining and by direct microscopic examination. Moreover, because of these difficulties in techniques, the literature is also somewhat confusing concerning the classification and differentiation of the stem cell of formed elements in the blood of many invertebrates.

Cuénot (1891) recognized the existence of only one basic class of granular blood cell in gastropod molluscs. Drew (1910), George and Ferguson (1950), and Dundee (1953) have reported the presence of at least three blood cell types in several species of molluscs. For the most part, all of the observations of the workers listed above were made on cover slip preparations with the use of ordinary light microscope.

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The classification of blood cell types in Crustacea also presents a confusing picture of blood cell morphology. Lochhead and Lochhead (1941) reported that only one basic class of blood cell and a phagocytic storage cell type were present in the blood of Artemia and Branchipus. Halliburton (1885) stated that there were two blood cell types in the crustaceans he studied. Recently George and Nichols (1948) made an extensive study of unstained and stained crustacean blood and reported that two general classes of blood cells could be detected in several species of crabs and crayfish. These they designated as cells with refractile granules and hyaline cells. The two general classes were further subdivided into four cell types and intermediates as (a) lymphoid cells; (b) colorless, semi-hyaline thigmotactic amoebocytes; (c) cells with coarse refractile granules and (d) cells with small refractile granules.

It is the purpose of this investigation to study the histological characteristics of the formed elements in the blood of the crayfish, blue crab and lobster in the living state by the use of the phase microscope. The formed elements in the blood of the same animals that have been fixed and stained will then be studied with the ordinary light microscope for comparative purposes.

## Materials and Methods

Adult female and male crayfish of the species *Cambarus bartoni* were obtained from the Carolina Biological Supply Company, Elon College, North Carolina; adult female and male lobsters, *Homarus americanus,* from the Lobster Pot Company of Washington, D. C., and adult female and male blue crabs, *Callinectes sapidus,* were caught by the writer in the Rappahannock River, near Urbanna, Virginia.

The blood was obtained by cutting off the tips of the claws with scissors and collecting a drop on a coverslip. The vaseline sealed coverslip preparation was immediately examined under oil immersion, using dark contrast and B minus phase objectives. In such preparations the cells remained alive and active and were studied for varying periods of time up to several hours.

The lymphatic tissue was obtained from all animals by removing the dorsal surface of the cardiac stomach. The material was fixed in 20% formalin in Ringer's solution. Bouin's was also used as a fixative for some tissues, but proved unsatisfactory. The heart was

also removed after both ends had been tied off to avoid loss of blood. Fixation was the same for the hearts.

Sections of the lymphatic tissue and heart were cut at 5$\mu$ and stained with Gomori's Chrome-Alum Hematoxylin Phloxin B. (Gomori, 1941). The preparations then were studied under oil immersion using the ordinary light microscope.

## Results

In the present work, the following criteria were used for the determination of blood cell types: the amount of cytoplasm, the presence or absence of refractile, cytoplasmic granules and the size of these granules. These criteria were applied mainly to living cells studied with the phase microscope. Although a comparison of the findings in live material with stained preparation was attempted, the following distinct cell types can be identified in all three of the crustacea studied.

1. *Lymphoid cell type*—

(a) In fresh unfixed and unstained blood preparations these cells are variable in form, being round, oval or spindle-shaped, with the majority tending to be round or oval when observed immediately after withdrawal from the animals. This cell type measures on the average 6$\mu$ x 7$\mu$ in the blue crab; 8$\mu$ x 11$\mu$ in the crayfish and 11$\mu$ x 13$\mu$ in the lobster. The nucleus occupies approximately three-fourths of the cell body and shows a distinct nuclear membrane. The nucleus appears to have either a longitudinal or a transverse furrow or occasionally several such furrows. The nuclear furrows are prominent in the crayfish, but less distinct in both the blue crab and lobster (Plate 1 ,Fig. 1, 2, 11). A few granules are scattered in the nucleus, but most of them form a distinct inner layer at the periphery of the nuclear membrane. The granules in the nucleus of the lymphoid cell of the lobster are larger than those of the crayfish and blue crab. The scanty cytoplasm contains a variable number of fine to medium sized granules and also one to two vacuoles. In many cells of this type the vacuoles contain granules in Brownian motion. After a lapse of several minutes this cell type may form one to two sharp or blunt pseudopodia and also several smaller projections extending from the pseudopodia (Plate 1, Fig. 3). The pseudopodia and their finer projections disappear and reappear irregularly. All cells of this type

## PLATE 1

### *Explanation of Figures*

1. *Cambarus bartoni*: Lymphoid cell showing a nucleus with a distinct longitudinal furrow, immediately after withdrawal of blood. The smaller oval body below this cell type is the nucleus of a disrupted explosive refractile granulocyte. X 800.
2. *Homarus americanus*: Lymphoid cell showing an oval nucleus, large nuclear granules and pseudopodium. 20 minutes. X 800.
3. *Homarus americanus*: Lymphoid cell showing pseudopodium with several fine projections. 30 minutes. X 800.
4. *Cambarus bartoni*: Lymphoid cell showing nucleus with several nuclear furrows. 15 minutes. X 800.
5. *Cambarus bartoni*: Lymphoid cell showing a filamentous pseudopodium containing three vacuoles at its distal end. The small granules in the three vacuoles were observed to be in Brownian motion. 15 minutes. X 800.
6. *Cambarus bartoni*: Lymphoid cell showing a small vacuole near the lobose pseudopodium. 10 minutes. X 800.
7. *Cambarus bartoni*: Monocyte cell showing slightly indented nucleus and large nuclear granules, immediately after withdrawal of blood. Note the three distinct vacuoles in the cytoplasm of the cell. X 800.
8. *Callinectes sapidus*: Explosive refractile granulocyte (large granule type) showing typical elongated refractile granules, an indistinct nuclear outline and pseudopodia. 20 minutes. X 900.
9. *Cambarus bartoni*: Monocyte cell showing a horseshoe-shaped nucleus, cytoplasmic granules and a distinct cytoplasmic vacuole, immediately after withdrawal of blood. X 800.
10. *Cambarus bartoni*: Monocyte cell showing numerous granules, kidney-shaped nucleus, distinct vacuole at the indented part of the nucleus and a pseudopodium at each axis of the cell. 45 minutes. X 800.
11. *Callinectes sapidus*: Lymphoid cell showing numerous granules and large vacuole in terminal part of pseudopodium. 15 minutes. X 900.
12. *Cambarus bartoni*: Lymphoid cell showing a filamentous pseudopodium. 30 minutes. X 800.

*Legend*: Photomicrographs of blood cells in unfixed and unstained blood of the crayfish, blue crab and lobster as observed with the phase microscope immediately after withdrawal of blood or after time as indicated.

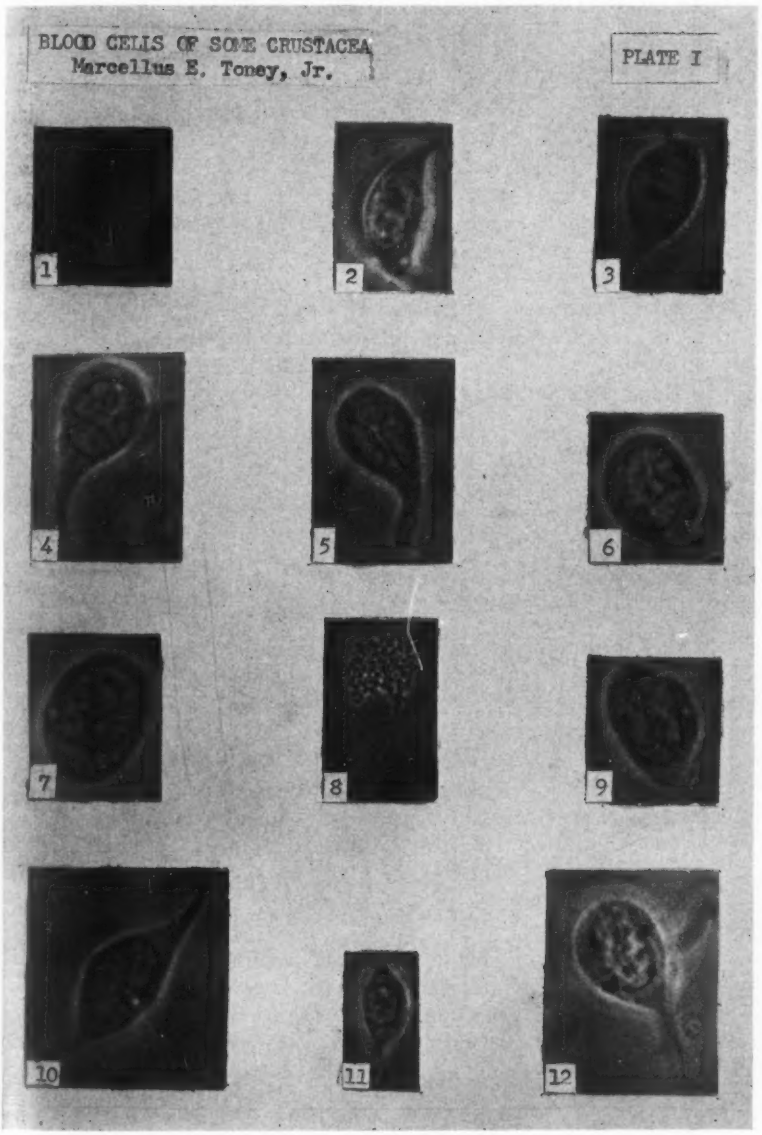
BLOOD CELLS OF SOME CRUSTACEA
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PLATE I
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exhibit amoeboid movement, but it appears to be more active in the crayfish than in the blue crab and lobster. The vacuoles that contain granules in Brownian movement are usually seen near the distal end of the pseudopodium or in a proxial position prior to the extension of the pseudopodium from the surface of the cell (Plate 1, Fig. 5). This cell type remains intact and active for several hours.

(b) In fixed stained heart and lymphatic tissues, these lymphoid cells are similar in form to those seen in fresh unfixed and unstained blood preparations. The round or oval nucleus occupies most of the cell and stains a medium blue purple. The nuclear membrane is distinct and more or less wrinkled. The nucleus of this cell type, in stained preparations of crayfish blood, exhibits a less distinct nuclear furrow than it does in fresh blood preparations and this is also true for the blue crab and lobster. A nucleolus is visible in most of these cells.

The scanty cytoplasm stains a light bluish purple and contains fine to medium bluish purple granules that vary in number from few to many. The cyptoplasm also contains several small vacuoles. Some of the cells exhibit what appears to be a pseudopodium at one axis of the cell body.

2. *Monocyte cell type—*

(a) In fresh unfixed and unstained blood preparations, this cell type is more or less similar to the lymphoid type of cell. Its average size is $6\mu$ x $7\mu$ in the blue crab, $7\mu$ x $9\mu$ in the crayfish and $7\mu$ x $10.8\mu$ in the lobster. This is the least common type detected in the fresh blood preparations of all animals.

The most prominent morphological characteristic of this cell type in the crayfish is the presence of a distinct horseshoe or kidney-shaped nucleus (Plate 1, Fig. 10). In the blue crab and lobster the nucleus also exhibits a horseshoe form, but is less distinct than it is in the crayfish. In many cells of this type numerous fine cytoplasmic granules are observed.

Other morphological characteristics of this cell type, including the presence of pseudopodia at various times, are similar to the lymphoid type. A nucleolus is visible in some of these cells.

(b) In fixed stained heart and lymphatic tissues, this cell type is generally similar to the same type found in fresh blood preparations. The nucleus stains bluish purple and also the chromatin granules.

The cytoplasm stains less intensely than that of the lymphoid cell and contain numerous fine purple orange granules.

3. *Explosive refractile granulocyte*—(Large refractile granule type.)

(a) In fresh unfixed and unstained blood preparations, these cells are variable in form, being round, oval or irregular, with the majority tending to be round or oval immediately after withdrawal from the animals. The average size is 14$\mu$ x 14$\mu$ in the blue crab, 15$\mu$ x 18$\mu$ in the crayfish and 16.8$\mu$ x 25.2$\mu$ in the lobster. This cell type is the most numerous in all fresh blood preparations, averaging 10-15 times as many in a drop of blood as any one of the other types.

Immediate examination of this type reveals a round or oval cell with numerous large, refractile cytoplasmic granules. These granules exhibit slight Brownian movement at this stage. Their shape is round or oval in the crayfish; elongated in the blue crab and very irregular in the lobster (Plate 2, Fig. 4). The average diameter of the large, refractile granules is 0.7$\mu$ in the blue crab; 2$\mu$ in the crayfish and 1$\mu$ in the lobster.

After periods of time varying up to several hours, this cell type exhibits a number of morphological changes. The large, refractile granules increase their Brownian movement and this is followed by a progressive disintegration resulting in the production of finer granules. A round or oval nucleus becomes visible after several large, refractile granules disintegrate. The nuclear membrane appears more distinct at this time. Several blunt or filamentous pseudopodia also become visible on the cell surface at this stage. As the cell becomes more elongated and fewer large, refractile granules remain visible, the cytoplasm exhibits two to six large clear areas which gives the cell an extremely vaculated appearance (Plate 2, Fig. 1). The nuclear details also become more evident at this time.

A number of large, irregular granules, presumed to be chromatin granules are seen scattered in the nucleus and along the periphery of the nuclear membrane. A nucleolus is visible in many of these cells at this time.

After the above morphological changes occur, this cell type exhibits a more or less abrupt "explosive" phenomenon. The peripheral surface of the cell appears to erupt and a network of numerous short and long fine projections is produced (Plate 2, Fig. 3). A large number of fine granules resulting from the disintegration of the large, refrac-

## PLATE 2

### *Explanation of Figures*

1. *Carbarus bartoni*: Explosive refractile granulocyte (large granule type) showing smaller granules that resulted from the partial disintegration of large refractile granules. Note the extremely vacuolated appearance of this cell and the indistinct outline of the nucleus. 30 minutes. X 800.
2. *Cambarus bartoni*: Explosive refractile granulocyte (large granule type) showing a few large refractile granules, pseudopodia and outline of nucleus. 15 minutes. X 800.
3. *Cambarus bartoni*: Explosive refractile granulocyte (large granule type) showing nucleus, pseudopodia with fine projections and extremely small granules that resulted from the complete disintegration of the large refractile granules. 45 minutes. X 800.
4. *Cambarus bartoni*: Explosive refractile granulocyte (large granule type) containing large refractile granules immediately after withdrawal of blood. X 800.
5. *Homarus americanus*: Explosive refractile granulocyte (large granule type) showing irregular shaped refractile granules, blunt pseudopodia and outline of nucleus. 30 minutes. X 800.

*Legend*: Photomicrographs of blood cells in unfixed and unstained blood of the crayfish, blue crab and lobster as observed with the phase microscope, immediately after withdrawal of blood or after time as indicated.

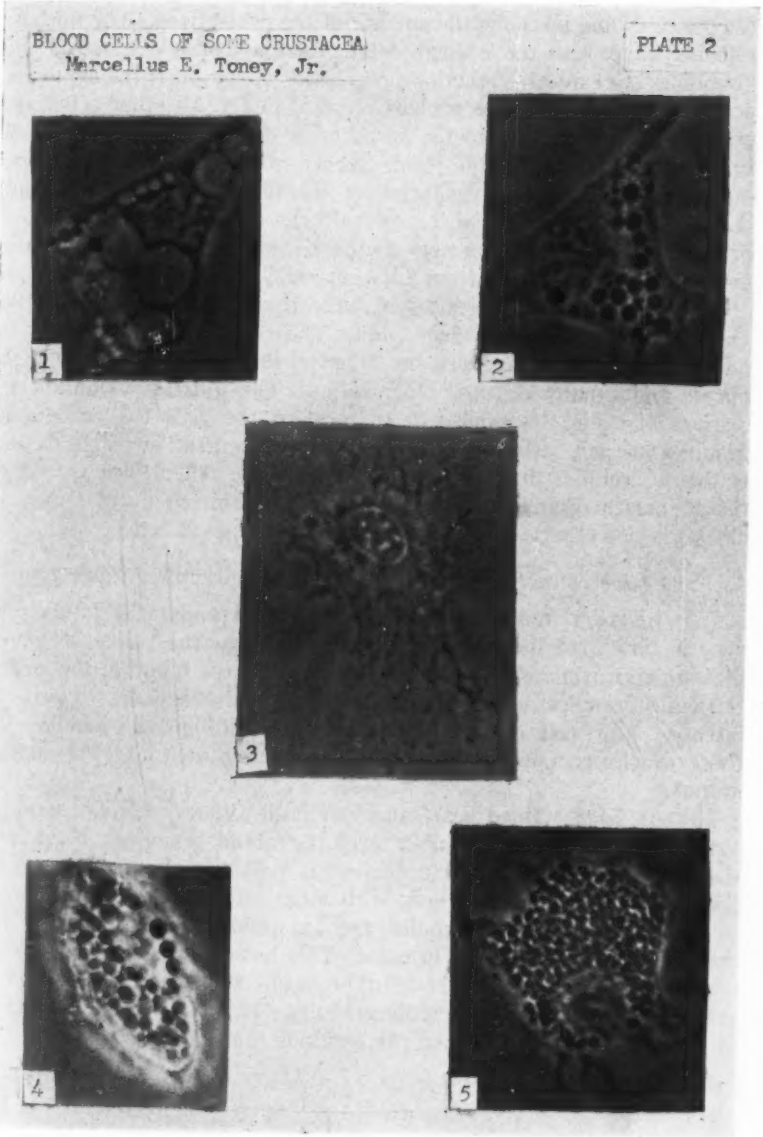
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PLATE 2
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tile granules line up along the surface of the projections. The nucleus remains intact and the nuclear details become more distinct as the "bubbling" or explosive reaction progresses. However, if the explosive action continues, only the nucleus remains intact, all other cell components are disrupted into the surrounding medium, leaving the cell in an almost unrecognizable form. Such forms occur, in all instances, during the process of coagulation of the blood. It is also evident that during coagulation, where several cells of this type are in close proximity to each other, a network consisting of long and short fine projections and granules from adjacent cells is produced.

(b) In fixed stained heart lymphatic tissues, the large, refractile granules stain a bright orange pink. The round or oval nucleus is not visible in all of the cells, but when visible, the nucleus is bluish purple and usually eccentric in position. The nuclear membrane is distinct and slightly wrinkled. In most of the cells the chromatin granules are not visible due to the heavy concentration of the large, refractile granules that stain orange pink in the cell. However, when the chromatin granules are visible they stain bluish purple. A nucleolus is not observed in the nucleus of the stained cells.

4. *Explosive refractile granulocyte*—(Small refractile granule type.)

(a) In fresh unfixed and unstained blood preparations this cell type is similar to the preceding type except that the refractile granules are approximately one-fourth the size of those found in the large, refractile granulocyte. The explosive action also appears to be less intense. This cell type exhibits morphological changes similar to those which occur in the explosive granulocytes with large, refractile granules.

(b) In fixed stained heart and lymphatic tissues, this cell type is again similar to the cells with large, refractile granules. However, the nuclear details where the nucleus is visible is slightly more distinct here than in stained cells with large refractile granules. Some of the small refractile granules are basophilic here in addition to those that are orange pink in color. This basophilic appearance may represent a transition in this cell type before it becomes an explosive granulocyte with large, refractile granules. This type is more difficult to detect in fixed and stained preparations than in fresh ones.

TABLE 1
Summary of cell types present and their morphological characteristics.

| Cell Types | Blue Crab | Crayfish | Lobster |
|---|---|---|---|
| 1. *Lymphoid cell*— | | | |
| a. Average cell size. | 6μ x 7μ | 8μ x 11μ | 11μ x 13μ |
| b. Nuclear furrow. | More or less indistinct. | Very distinct. | More or less indistinct. |
| c. Amoeboid movement. | Moderately active. | Very active. | Moderately active. |
| 2. *Monocyte cell*— | | | |
| a. Average cell size. | 6μ x 7μ | 7μ x 9μ | 7μ x 10.8μ |
| b. Nuclear form. | Slightly indented. | Horseshoe or very indented. | Slightly indented. |
| c. Amoeboid movement. | Moderately active. | Very active. | Moderately active. |
| 3. *Explosive refractile granulocyte.* | | | |
| (Large refractile granules.) | | | |
| a. Average cell size. | 14μ x 14μ | 15μ x 18μ | 16.8μ x 25.2μ |
| b. Average size of refractile granules. | 0.7μ | 2μ | 1μ |
| c. Shape of refractile granules. | Elongated. | Oval. | Irregular. |
| 4. *Explosive refractile ganulocyte.* | | | |
| (Small refractile granules.) | | | |
| a. Average size of refractile granules. | 0.17μ (Average size of several granules measured together.) | 0.5μ (Average size of several granules measured together.) | 0.25μ (Average size of several granules measured together.) |

## Discussion

The correlation of results of the present investigation with previous observations on blood cell morphology in Crustacea is made difficult by the fact that all previous investigators used morphological characteristics identified by the ordinary light microscope or by the behavior of the blood cells in drop studies observed with the same type of microscope. In all such reports the morphological details are scanty. Therefore, it is impossible to correlate, in all cases, the cell types found here with those of Halliburton (1885), Hardy (1892), Tait and Gunn (1918), Lochhead and Lochhead (1941), and George and Nichols (1948). However, the findings in the present work are in partial accordance with some of the cell types observed by the above mentioned workers. The present work attempts to classify cell types mainly on the basis of strict morphological characteristics such as nuclear shape and size; nuclear-cytoplasm ratio; granules; pseudopod formation and to a lesser degree also functional activity of the cell types.

The cell designated here as the lymphoid type, corresponds, to some degree, with the pale amoeboid cell of Halliburton's (1885) classification of blood cells in the crayfish and lobster. The pale amoeboid cell of Halliburton (1885) contained fine processes similar to the ones that are present in the lymphoid cell in the present work. However, the description of the amoeboid cell type of Halliburton lacks morphological detail, so that any further correlation between this cell type and the lymphoid cell here is quite difficult. Hardy (1892) working with Astacus and Daphnia reported the presence of two cell types which he designated as (a) an explosive cell and (b) an eosinophilic refractile granular cell. The explosive cell of Hardy was an oval cell that contained few to many cytoplasmic granules; an oval nucleus; and elongated cytoplasmic processes. The eosinophilic refractile granular cell of Hardy contained numerous eosinophilic granules and was amoeboid in movement. This cell also exhibited pseudopodia, but there was no evidence of any change in its morphological characteristics after varying periods of examination. It seems likely that some of the cells classified as explosive cells by Hardy were probably of the type designated as lymphoid in the present work, and the eosinophilic refractile granular cells of Hardy corresponded more to the explosive refractile granulocyte described here. It appears that Hardy based the classification of his explosive

cell mainly on its ability to produce elongated processes; he did not mention any abrupt disruption of the cytoplasm. Tait and Gunn (1918) also working with Astacus, demonstrated a blood cell type which they classified as an amoebocyte. It contained a granular cytoplasm, possessed pseudopodia, was constantly changing in shape and amoeboid in movement. In the above characteristics Tait and Gunn's amoebocyte also corresponds in general with the lymphoid cell type of the present work. These authors also reported another cell type in the blood of Astacus, which they called a thigmocyte. The classification of this cell as a distinct type was based mainly on its ability to emit fine pointed processes. However, the presence of such processes was observed in the lymphoid cell in the present work. George and Nichols (1948) reported the presence of a hyaline lymphoid type of cell in the blood of the fresh-water crayfish, blue crab and other related species. This cell type is similar to the lymphoid cell in the present work. There are, however, differences since the lymphoid cell type described here possesses several vacuoles which contain fine granules in Brownian movement. These were not reported by George and Nichols for the same cell type. The presence of pseudopodia beset with finer projections in the lymphoid cell type in the present work is another feature not mentioned for the hyaline lymphoid cell of George and Nichols. According to these authors, the hyaline lymphoid cell contains either a relatively large spherical or indented nucleus with nucleolus, similar to the lymphoblasts of vertebrates. In the present work the lymphoid cell contains, especially in the crayfish, a round or oval nucleus with one nuclear furrow or with several furrows. It was obviously impossible for previous investigators to detect all of the above morphological characteristics of the lymphoid cells seen in fresh unfixed and unstained blood, unless phase microscopy and controlled conditions had been employed in the examinations.

According to George and Nichols (1948), some of the hyaline lymphoid cells had the general appearance of monocytes of vertebrates. We also detected cells that are similar to monocytes. However, this cell possesses such definite morphological characteristics of monocytes such as the presence of numerous cytoplasmic granules and a horseshoe or kidney-shaped nucleus, that this cell is designated as a distinct cell type in the present work.

No distinct cell types were observed here that corresponded to the

thigmocyte of Tait and Gunn (1918) or the semi-hyaline thigmotactic amoebocyte of George and Nichols (1948). The semi-hyaline thigmotactic amoebocyte shows variation in size and form and contained few to many cytoplasmic granules. They also possess vacuoles that contain small granules in Brownian movement. The cells are highly thigmotactic and were observed to flatten out rapidly in fresh blood to form thin films of protoplasm with slender pseudopodia. However, all of the above cytological characteristics of the thigmocyte and amoebocyte of Tait and Gunn (1918) and the semi-hyaline thigmotactic amoebocyte of George and Nichols (1948) are observed in the lymphoid cell type in the present work.

As stated previously, the explosive granulocyte containing large, refractile granules designated here, represents the highest percent of all cell types observed in the fresh blood preparations of all animals. It appears that this cell type and the explosive granulocyte containing small refractile granules, are main types involved in the active coagulation of the blood. It is possible that the changing morphology that occurs in these cell types represent stages in preparation for the active coagulation of the blood. No other cell types are observed to take an active part in the coagulation of the blood in the animals studied. In general, the findings in the present work for the same cell types stated above are in accordance with the observations of George and Nichols.

Grégoire (1953) working with sixty-one species of insects observed a cell type in the blood of these animals that was similar to the semi-hyaline amoebocyte in Crustacea. This cell, designated as a hyaline hemocyte, is an oval cell that possesses a round or oval nucleus and exhibits a hyaline cytoplasm containing a few dark granules. This cell type also shows morphological changes after varying periods of time, similar to the explosive refractile granulocytes in the present work. It was observed to rupture and form a circular area of granules around the indistinct cell outline as a result of the rupturing. Several long thin pseudopodia were also formed. The pseudopodia and granules form a network which, according to Grégoire, releases a substance that leads directly to the coagulation of the blood. Although the general behavior of the explosive refractile granulocyte in the present work is in accordance with the hyaline hemocyte of Grégoire in regard to the process of coagulation of blood, these two cell types cannot be correlated in so far as their morphological characteristics are concerned.

## Summary

The histology of the formed elements in the blood of the crayfish, blue crab and lobster was studied in the living state by the use of the phase microscope and the following blood cell types were distinguished: (a) lymphoid cells, (b) monocytes, (c) explosive refractile granulocytes with large refractile granules and (d) explosive refractile granulocytes with small refractile granules.

Fixed and stained blood cells in the heart and lymphatic tissues were also studied. All of the blood cell types that were observed in the living state were identified in the fixed and stained preparations. However, many of the morphological characteristics observed in the blood cells in the unfixed and unstained preparations were not visible in the stained sections. These included the absence of pseudopodia in most of the stained cells, less distinct nuclear furrows, and scanty nuclear details. Only observations of cells in the living state also revealed such features as amoeboid movement, Brownian movement of the granules in the vacuoles of the lymphoid and monocytes and the various cytological changes of the explosive refractile granulocytes. In the lymphatic tissue and in some of the blood spaces there were observed additional cell types that were not detected in the unfixed and unstained preparations or in the stained blood cells of the heart. There were intermediate cell types that showed variation in size, form and staining characteristics and exhibited more or less lymphoid morphological characteristics. Therefore, it is possible that the lymphoid cell types observed in the lymphatic tissue represented the stem cell for the differentiation of all other blood cell types observed in the animals studied.

The findings in the present investigation are in partial accordance with the classification of George and Nichols (1948).


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*Growth*, 1958, **22**, 51-61.

# ADDITIVE EFFECT OF ASCORBIC ACID AND GLUCOSE ANALOGS ON EXPERIMENTAL CANCER AND NUCLEIC ACIDS IN TUMOR TISSUE*

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In our previous papers (14, 15), we reported that 2,3-enediol-D-glucohepton-1,4-lactone, an antagonist of ascorbic acid, when added to a Sherman-LaMer ration in 1 or 2 per cent, considerably retarded the Crocker rat carcinoma and mouse adenocarcinoma E 0771. Added to a Sherman-LaMer ration in 0.75 per cent, it did not affect the incidence of lympathic leukemia in the Ak-lm strain but prolonged the life span of leukemic mice by 26.8 per cent (16, 17, 18). The analog decreased the total and nuclear DNA contents of Crocker rat carcinoma (19, 20). 2-Deoxy-D-glucose, an analog of glucose competed with glucose in the yeast fermentation, according to Cramer and Woodward (3). Woodward and Hudson (24) found that the analog of glucose inhibited glycolysis of rat tumor slices and the growth *in vivo* of Flexner-Jobling and Walker 256 carcinoma. Sokoloff *et al.* (21) reported that 2-deoxy-D-glucose added to a meat ration in the amounts of 2% or 5% inhibited considerably the growth of Crocker rat carcinoma. In their more recent paper (22), they showed that the analog also inhibited the growth of rat carcinoma G-175 when added to the meat ration, and when injected subcutaneously in the doses of 500 mg/kg wt/day for seven days to mouse sarcoma 180 and rat carcinoma G-175. The total and nuclear DNA content in rat carcinoma G-175 was decreased by administration of the analog.

Laszlo *et al.* (6) disclosed that 2-deoxy-glucose and 2-deoxy-galactose were potent glycolytic inhibitors of tumor cells in every instance they studied. Glycolytic inhibition, as high as 50 per cent was achieved at a dosage level of 50 mg/kg wt. given in a single intravenous leukemic infusion. Oral administration of the analog to ad-

* Aided by grants from the Damon Runyon Memorial Fund for Cancer Research and the Marcia Tucker Foundation, New York, N. Y.

vanced L-1210 mouse leukemia results in the prolongation of the life span by 20-30 per cent and reduction in tumor size.

In our present study, the effect of the combined application of the analogs of ascorbic acid (D-glucoascorbic acid) and of glucose (2-deoxy-D-glucose) on mouse sarcoma 180 and Crocker rat carcinoma was investigated and the alteration in the total and nuclear DNA content of Crocker carcinoma was estimated.

## Material and Methods

The British brown-white strain of rats, 188-AW, originally obtained from the Crocker Laboratory at Columbia University with transplanted Crocker carcinoma and mouse sarcoma 180 were used in this study. The combined antitumor activity of 2-deoxy-D-glucose (2-DG) and glucoascorbic acid (D-GA) was investigated both by oral administration and subcutaneous injections. Both species of animals were placed on an exclusive meat diet, consisting of fat free chopped beef meat for three days prior to transplantation. The meat diet was well tolerated by the animals and by itself did not affect the growth of tumors to any significant degree. The analogs were added to the meat ration five days after tumor transplantation when the growth reached a size of 1.0-2.0 cm. in diameter. The subcutaneous injection of the analogs was applied in the seven-day standard test. Here again the therapy was started five days after transplantation and not 24 hours after it as is the usual practice of such tests. Thus we were dealing with tumors already in the process of active growth. The tumors used for the determination of nucleic acid were about 2 cm. in diameter.

A modified method of Barnum (1) and Payne *et al.* (11) as described by us in our previous publication (22) was used for the extraction and separation of DNA and PNA. The technique of Schneider (13) was employed for the estimation of DNA and PNA in the tumor tissue. The estimation of the nuclear DNA was made according to the procedure recommended by Lessler (7) and described in detail in our previous paper (20).

## Results

*Toxicity of 2-DG and D-GA.* As reported previously by us (14), one per cent of D-GA added to the ration did not produce any ill effect on rats after eight weeks of dieting. 2-DG added to the ration

of adult rats in the amount of two per cent was well tolerated by animals. No loss in weight was noted as compared with the control animals. However, the ration containing two per cent of 2-DG considerably inhibited the growth of young rats, up to 20 per cent as against the control. Both analogs produced no toxic effect when injected subcutaneously or intraperitoneally to mice and rats, in a dose of 500 mg/kilo weight for deoxyglucose and 300 mg/kilo weight for d-glucoascorbic acid, for seven days or more.

On the basis of this data, previously reported, 40 rats, average weight 195 grams, all males, were divided into two groups. Group I was placed on an isocaloric meat diet for twenty days. Group II was placed on an isocaloric meat diet to which two per cent 2-DG and one per cent D-GA was added. No apparent ill effect was observed in the treated group. The average weight for 20 rats in the control group went up from 192 grams to 202 grams in twenty days or an increase of about 5 per cent. In the treated group, the increase in the average weight amounted to 2.2 grams, from 196 to 198.2 grams, or about 1 per cent.

*Anti-tumor activity of the combined analogs.* Eighty rats, average weight 172 grams, all males, were divided into four groups. Group I —control, isocaloric meat ration; Group II—meat ration with one per cent D-GA added; Group III—meat ration with two per cent 2-DG added; and Group IV—meat ration containing one per cent D-GA and two per cent 2-DG. The animals of Groups II, III and IV were placed on the exclusive meat ration for three days before the tumor, Crocker carcinoma, was transplanted. On the fifth day after the transplantation, and when the tumors had reached the average size of 2.0 cm. in diameter, the rations were changed to the ones containing the analogs. The tumors were measured every five days. Four weeks after transplantation, the rats of all four groups were killed, the tumors removed and weighed. The average tumor weight for Group I was 21.3 g.; Group II—13.8 g.; Group III—9.4 g.; and Group IV—3.5 g., with four cases of complete regression of the growth. Chart I summarizes the results of this experiment.

The seven-day standard tests were conducted on 40 rats divided into four groups, all kept on a meat ration for four days before transplantation of the tumor. The treatment was started on the fifth day after transplantation. Group I—control; Group II—subcutaneous injections, 150 mg/kilo wt./day/rat of D-GA (buffered) for seven

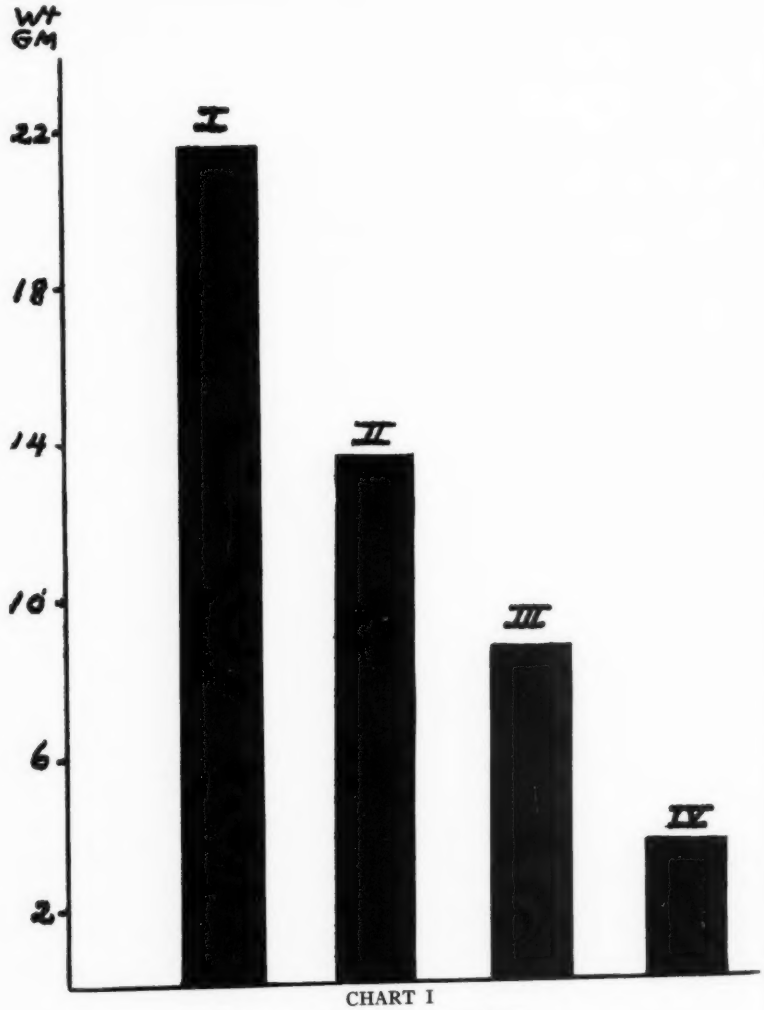


CHART I

Growth of Crocker rat carcinoma

Group I: Control, 20 rats, isocaloric meat diet
Group II: 20 rats, isocaloric meat diet supplemented with 1% of D-GA
Group III: 20 rats, isocaloric meat diet supplemented with 2% of 2-DG
Group IV: 20 rats, isocaloric meat diet supplemented with 1% of D-GA and 2% of 2-DG.

Tumor weight in grams.

days; Group III—250 mg/kilo wt./day/rat of 2-DG for seven days; Group IV—150 mg. D-GA (buffered) and 250 mg. 2-DG per kilo body weight for seven days. After the tests were terminated, the rats were killed and the tumors weighed. The average weight for Group I, control was 8.4 g.; Group II—7.2 g.; Group III—5.6 g.; and for Group IV—4.1 g. Or the combined administration of the two analogs reduced the growth by 50 per cent as against the control tumors. Analyzing the data of these two experiments, a four week feeding test and the seven day standard test (Chart II) and comparing the end results, one may say that the analogs were apparently more effective when administered for a longer period of time rather than for only seven days. Similar results were obtained with mouse sarcoma 180.

Sixty mice were divided into four groups as in the previous experiments. The subcutaneous injections of the analogs were started three days after transplantation of sarcoma 180 and were given for seven consecutive days. Group I—control; Group II—150 mg/kilo wt/day/mouse D-GA; Group III—250 mg/kilo wt/day/mouse 2-DG; and Group IV—150 mg/kilo wt/day/mouse d-GA and 250 mg/kilo wt/day/mouse 2-DG. The tumors were excised and weighed. The average weight for Group I was 1950 mg; for Group II—1640 mg; for Group III—1430 mg; and for Group IV—1190 mg. The results, as presented in Chart III, show that the combined treatment with both analogs inhibited the tumors by reducing their size by about 40 per cent as compared with control.

*Total PNA and DNA Content of Crocker Rat Carcinoma.* Forty male rats, average weight 192 gm., were divided into four equal groups and placed on an isocaloric meat ration. Group I—control; in Group II, the ration was supplemented with 1 per cent of D-GA; and in Group III with 2 per cent of 2-DG. Group IV was fed a ration containing both analogs, 1 per cent of D-GA and 2 per cent of 2-DG. Crocker carcinoma was transplanted to all animals after three days on the diets. The tumors were removed 8 days after transplantation when they were approximately 0.75 inches in diameter. 15-16 hours before excision of the tumors, food was withdrawn from the animals. The total PNA and DNA of tumor tissue was estimated according to Schneider's technique (13). The data, presented in Table I, indicated that d-glucoascorbic acid did not significantly affect the PNA content of the tumor tissue, while 2-deoxyglucose brought it down con-

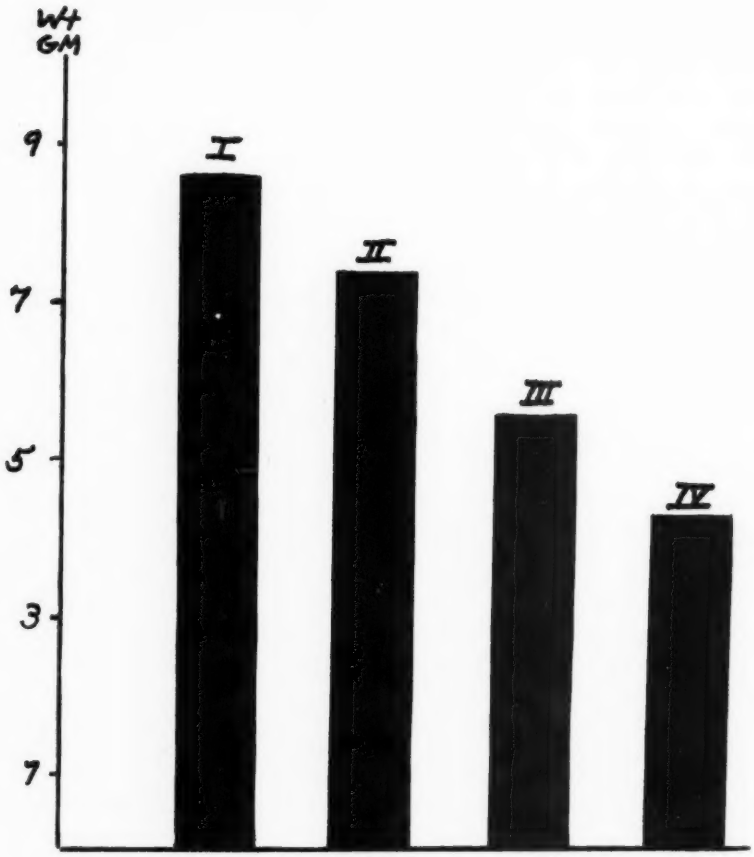


CHART II

Growth of Crocker rat carcinoma
Seven day standard test
Subcutaneous injections

Group I: Control, isocaloric meat diet
Group II: 150 mg/kilo wt./day/rat of D-GA
Group III: 250 mg/kilo wt./day/rat of 2-DG
Group IV: 150 mg of D-GA and 250 mg of 2-DG per kilo wt./day/rat

Tumor weight in grams.

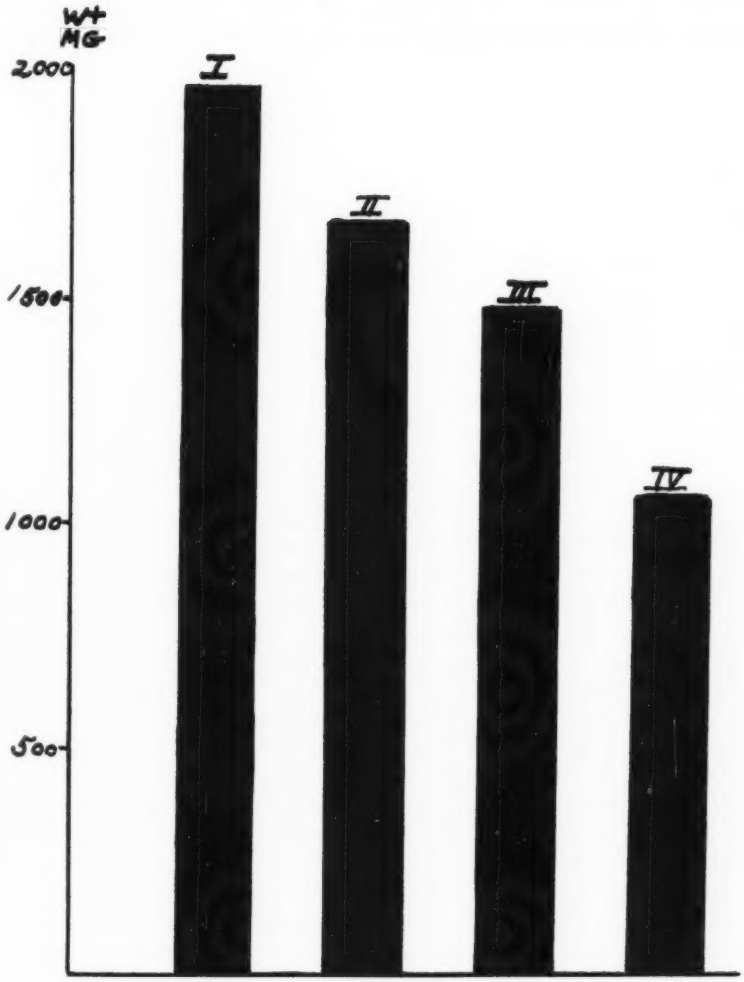


CHART III

Growth of mouse sarcoma 180

Seven day standard test

Subcutaneous injections

Group I: Control, isocaloric meat diet
Group II: 150 mg/kilo wt./day/mouse of D-GA
Group III: 250 mg/kilo wt./day/mouse of 2-DG
Group IV: 150 mg of D-GA and 250 mg of 2-DG kilo wt./day/mouse

Tumor Weight in Milligrams

TABLE I
Effect of D-Glucoascorbic Acid and 2-Deoxyglucose on the PNA and DNA Content of Crocker Rat Carcinoma

| Group | Supplement | No. Tumors | PNA per cent of dry wt. | DNA per cent of dry wt. | PNA/DNA Ratio |
|---|---|---|---|---|---|
| I | None | 5 | 2.46 ∓ 0.17 | 3.11 ∓ 0.21 | 0.79 |
| II | 1 per cent D-GA | 6 | 2.33 ∓ 0.23 | 2.34 ∓ 0.15 | 1.00 |
| III | 2 per cent 2-DG | 5 | 2.24 ∓ 0.11 | 2.23 ∓ 0.12 | 1.00 |
| IV | 1 per cent D-GA and 2 per cent 2-DG | 6 | 1.93 ∓ 0.18 | 1.67 ∓ 0.21 | 1.15 |

siderably. The combined effect of both analogs, however, seemed to be more pronounced than the one of 2-DG alone. The DNA content of the tumor tissue was decreased by both analogs separately or jointly applied, and there seemed to be an additive effect of such a combined administration of the two analogs.

*Nuclear DNA Content of Crocker Rat Carcinoma.* The nuclear DNA content of Crocker rat carcinoma was estimated with the Feulgen reaction. Forty male rats, average weight 177 g., were divided into four groups and treated along the same lines as in the previous experiments. The tumors were excised 8 days after transplantation at which time their size was about 0.75 inches in diameter. Table II summarizes the results of the estimation. The DNA nuclear content is expressed in arbitrary units which were calculated by multiplication of the extinction coefficient of the nucleus by the area.

The analysis of the data presented in Table II indicates that simultaneous feeding of the rats, bearers of Crocker carcinoma, with the two analogs reduced the nuclear DNA content of the tumor tissue by 25 per cent.

## Discussion

Woolley (25) postulated that d-glucoascorbic "is undeniably analog in structure to ascorbic acid." Sokoloff *et al.* (15) reported that the addition of 1-2 per cent d-glucoascorbic acid to a Sherman-LaMer diet brought the ascorbic acid concentrations of blood plasma, adrenal, liver and spleen close to zero in rats and mice without inducing diarrhea, loss of weight, or hemorrhage. The findings of Robertson, *et al.* (12) and Sokoloff *et al.* (15) seem to indicate that the low concentra-

TABLE II

Effect of D-Glucoascorbic Acid and 2 Deoxyglucose on the Nuclear Content in Crocker Rat Carcinoma

| Group | Supplement | No. Specimens* | No. Cells | (E) x (A)** Arbitrary Units |
|---|---|---|---|---|
| I | None | 5 | 70 | 4.05 — 0.10 |
| II | 1 per cent D-glucoascorbic acid | 6 | 100 | 3.66 — 0.12 |
| III | 2 per cent 2-deoxyglucose | 5 | 100 | 3.42 — 0.09 |
| IV | 1 per cent D-glucoascorbic acid and 2 per cent 2-deoxyglucose | 7 | 125 | 3.02 — 0.19 |

* Each specimen was taken from a different tumor.
** E—extinction coefficient.
A—nuclear area, in $D^2$.

tions of ascorbic acids in tumor tissue and blood plasma considerably inhibited the growth in guinea pigs, rats and mice. Miller *et al.* (8) found that the patients affected with Hodgkin's disease and kept on vitamin C-free diet for several weeks during the X-ray therapy, responded more favorably to it than the cases kept on a regular diet. Goldstein *et al.* (4) disclosed that a scorbutogenic diet induced changes in PNA and DNA content of the spleen and adrenals of guinea pigs and increased the PNA/DNA ratio. On the basis of his experiments *in vitro,* they concluded that ascorbic acid is involved in the formation of DNA from PNA. According to Nichol and Welch (9, 10); Broquist *et al.* (2); Hill and Scott (5); and Welch *et al.* (23), ascorbic acid, a reducing agent, enhanced the formation of the citrovorum factor (CFO) from folic acid (PGA). On the other hand, the investigations of Cramer and Woodward (3), Woodward and Hudson (24) and Laszlo *et al.* (6) pointed out the fact that 2-deoxyglucose is a powerful glycolytic inhibitor. In our present study, an attempt was made to attack cancerous growth by two analogs interfering differently with cellular metabolism. The results of our experiments suggest that there was an additive effect on tumor growth of these two analogs, resulting in its considerable but not complete inhibition, and in a substantial decrease in the total and nuclear DNA content in the tumor tissue. As long as these analogs are relatively non-toxic, yet moderately effective antitumor agents, they deserve further attention of the investigators in the cancer field.

## Summary

d-Glucoascorbic acid, analog of ascorbic acid, and 2-deoxyglucose, analog of glucose, added simultaneously to isocaloric ration of rats, inhibited considerably but not completely Crocker carcinoma. There seemed to be an additive antitumor effect in such a combined treatment.

d-Glucoascorbic acid and 2-deoxyglucose injected simultaneously to mice bearers of Sarcoma 180, for seven days, inhibited moderately the tumor growth.

The two analogs decreased, when administered simultaneously, the total and nuclear DNA content in Crocker carcinoma tissue. This effect was more pronounced than in the case when either of the analogs was administered separately.

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# AN INVESTIGATION OF BLOOD GLUTATHIONE LEVELS IN SHEEP

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THE relation of blood glutathione (GSH) level to growth and body size and the influence of nutritional environment on blood GSH level in beef cattle has been investigated by Kidwell *et al.* (1955, 1956). This paper presents an analysis of similar experiments with sheep. These studies were motivated by the practical problem of finding an objective indicator, amenable to early measurement, of an animal's subsequent rate and economy of gain and ultimate body size. The evidence of an association between GSH concentration and rate of cell proliferation, protein synthesis and adult size in various animal species has been reviewed in the publications cited.

## MATERIALS AND METHODS

Two experiments contributed to this investigation. The animals of the first experiment consisted of 48 cross bred (Rambouillet X Hampshire) feeder lambs. The lambs were born in March and April, 1957, on the University of Nevada Range Station in northeast Elko County and, with their dams, placed on irrigated grass and clover pasture at the Reno Station in June. They were weaned about three weeks prior to the start of the experiment. Twenty-four lambs of each sex (wethers and ewes) were allotted to twelve groups of four lambs each on the basis of sex and weight. The groups were randomly assigned to treatments in a $2 \times 2 \times 3$ factorial design. The treatments included two levels of diethylstilbestrol, zero and 0.8 mg. per pound of feed; two levels of chlortetracycline, zero and 10 mg. per pound of feed and three levels of animal fat, zero, three and six per cent of the ration. The entire ration was fed in the form of $\frac{3}{8}$ inch pellets. The twelve rations are presented in Table 1. Table 1 also illustrates the experimental design. The lambs were maintained in small indoor pens ($4' \times 8'$) in groups of two, a wether and ewe, during the experiment.

TABLE 1
Physical composition of experimental rations—percent.

| Ingredient | 10 mg. chlortetracycline/lb. feed | | | | | | None | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.8 mg. diethylstilbestrol/lb. feed | | | None | | | 0.8 mg. diethylstilbestrol/lb. feed | | | None | | |
| Fat | 0 | 3.0 | 6.0 | 0 | 3.0 | 6.0 | 0 | 3.0 | 6.0 | 0 | 3.0 | 6.0 |
| Barley | 19.5 | 16.5 | 13.5 | 19.5 | 16.5 | 13.5 | 19.5 | 16.5 | 13.5 | 19.5 | 16.5 | 13.5 |
| Alfalfa | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 | 80.0 |
| Salt | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |

TABLE 2
Means with 95% confidence interval and correlations among them for blood GSH, total cell count, hemoglobin, and hematocrit of twenty-four lambs.

| Item | Means | Product-Moment Correlations | | |
|---|---|---|---|---|
| | | Hematocrit | Hemoglobin | Total Cell |
| GSH, mg % | 32.2 ± 4.2 | 0.55** | .53** | 0.51* |
| Total cell, $mm^3$ | 8.0 ± 0.2 | 0.76** | 0.90** | — |
| Hemoglobin, gm % | 14.5 ± 0.4 | 0.93** | — | — |
| Hematocrit, % RBC | 39.3 ± 1.2 | — | — | — |

* 5% level of significance
** 1% level of significance

Feed economy data therefore, represent the averages of the two animals in each pen. Weights and blood samples were obtained initially and at the end of a 60 day feeding period.

In the laboratory it was convenient to analyze only 30 samples per day. Therefore, half the lambs were bled one morning and half the next. In order to estimate the repeatability of estimates determined from different blood samples drawn after a short time interval, the first twenty-four were again bled on the third morning.

Blood GSH, total cell count, hemoglobin and hematocrit and the six possible correlations among them were determined from the first twenty-four samples and are presented in Table 2. In view of the magnitude of these correlations it was decided to omit total cell count and hemoglobin from the remainder of the study.

In addition to blood GSH and hematocrit, blood fat was determined immediately before and after the feeding period. Glutathione was also determined in liver samples obtained at slaughter.

Blood fat was determined by the method of Allen, 1938. The remaining analytical procedures were those described by Kidwell *et al.*, 1955.

The purpose of the second experiment was to estimate the effect

of ambient temperature on blood glutathione level. Twenty-four aged (6 years or older) range Rambouillet ewes were selected at random from among the dams of the lambs. They had all weaned lambs about six weeks prior to the experiment, and had not yet been mated. The ewes were weighed, heart girth measured and blood samples drawn at mid morning with an outside temperature of 57° F. At 6 p.m. they were divided at random into two groups of twelve each; one group was placed in a constant temperature room at 85° F. and the other in a constant temperature room at 40° F. Blood samples were taken twelve hours later and the ewes run together outside during the day. At 6 p.m. half of the ewes from each of the two previous temperature treatments were placed in the high temperature room and the rest of the ewes in the low temperature room. Blood samples were taken twelve hours later. Analyses were completed within six hours each day. All blood samples were analyzed for GSH and hematocrit. There were, therefore, four temperature treatment groups of six ewes each: continuous high; high-low; low-high and continuous low. The data from both experiments were amenable to analysis of variance and correlation techniques as described by Snedecor (1956).

## Results and Discussion

### *Lamb Experiment*

No sex differences were found for any of the traits measured. Chanton (1932) observed higher blood GSH levels in the male than in the female of the domestic fowl. The data of Kunkle *et al.* (1954) suggested sex differences in blood GSH level in cattle.

The mean blood GSH of the twenty-four lambs bled the second morning was significantly higher than the mean of those sampled the first morning (38.5 vs. 32.2), but the hematocrit level and body weight were the same. Moreover, levels of blood GSH were significantly different when determined on the same lamb over a 48 hour interval, (32.2 vs. 35.4). The mean difference and 95% confidence interval was $3.2 \pm 1.5$. Each day duplicate blood samples from several randomly selected lambs were analyzed. In each instance duplicates agreed to the second decimal. It had previously been determined that the chemical method was quite accurate.

The correlation between two determinations on the same lamb 48 hours apart was 0.95, indicating a fairly constant change for all lambs. This suggestion is supported by the small standard error (0.7)

and coefficient of variation (0.97%). The hematocrit was constant when determined on the same lamb on different days. In view of the relative constancy of the difference, it appears valid to consider correlations involving blood GSH, but to compute them on an intra day basis.

Means, 95% confidence intervals and correlations among the variables measured in the entire group of 48 lambs prior to the start of the feeding period are presented in Table 3. Blood GSH is associated with blood fat and hemotocrit, but not with body weight. The absence of an association between blood GSH and body weight was also observed in cattle by Kidwell *et al.* (1955, 57). None of the remaining correlations were significant, although the correlation between hematocrit and blood fat approached significance. The means and correlations (Tables 2 and 3) among the variables are of similar magnitude of those observed in beef cattle by Kidwell *et al., loc. cit.*

Two of the several possible causes of the observed difference in mean blood GSH between days were investigated. The standard GSH solution used in the analysis was made prior to the determinations made on the first morning. Since the samples for any given day are read over a very short time interval, any changes in the standard could result in a difference of the type observed. The standard was rechecked and found unchanged.

Wide differences in outside temperatures were noted during the three mornings the samples were taken. It appeared possible that differences in ambient temperature might induce a fairly constant change in blood glutathione. Brody *et al.*, (1949) and Blincoe and Brody (1951) found blood creatinine to increase with increasing temperatures while non protein nitrogen, ascorbic acid and carbon dioxide combining

TABLE 3

Means 95% confidence interval and correlations among variables measured three weeks post weaning and immediately prior to a 60 day feeding period.

| Item | Mean | Product-moment correlations | | |
|---|---|---|---|---|
| | | Body Weight | Blood Fat | Hemato-crit |
| Blood GSH, mg % | 1. | .14 | .44** | .48** |
| Hematocrit, % RBC | 40.0 ± 0.7 | —.13 | .27 | — |
| Blood Fat, mg % | 87.0 ± 5.8 | .10 | — | — |
| Body Wt., lbs. | 87.2 ± 1.8 | — | — | — |

1. There was a significant difference between means of samples taken on successive days. Explanation in text.

** 1% level of significance

capacity decreased with increasing temperature. Blood calcium, sodium, potassium, phosphorus, uric acid, creatine, ketones, hemoglobin, red cell count, white cell count and differential count were not influenced by temperature changes. The ewe experiment was conducted to estimate the influence of ambient temperature and is described later in the paper.

At the conclusion of a 60-day feeding period the lambs were weighed, heart girth measured and blood samples drawn for determination of GSH, hematocrit and blood fat. GSH was also determined from samples of liver tissue. Changes in GSH, hematocrit, weight, blood fat and feed economy, expressed as pounds of feed per pound of gain, were also determined. Analysis of variance indicated no difference in GSH or hematocrit between the groups bled on two successive days, and no sex or treatment effects on any of the variables measured. The entire group of 48 lambs was, therefore, considered as a single population for the purpose of determining correlations among the variables.

The means and 95% confidence intervals and correlations among the variables measured at the conclusion of the feeding period are shown in Table 4. Except for a low positive correlation with hematocrit, blood GSH is not associated with any of the other variables. An association between GSH and heart girth was observed with cattle (loc. cit.), and with the ewes employed in this study. An explanation is not immediately apparent for the significant correlation between blood GSH and blood fat prior to the feeding period and the absence of a relation at the conclusion.

There is a significant negative correlation between blood fat and weight at the conclusion of the feeding period, whereas there was no

TABLE 4
Means, 95% confidence interval and correlations among variables measured at conclusion of a 60 day feeding period.

| Item | Mean | Product-moment correlations | | | | |
|---|---|---|---|---|---|---|
| | | Heart Girth | Weight | Liver GSH | Hemato-crit | Blood Fat |
| Blood GSH, mg % | 36.4 ± 2.6 | —.05 | .07 | —.02 | .30* | —.14 |
| Blood fat, mg % | 107.9 ± 8.9 | .21 | —.29* | —.01 | .04 | — |
| Hematocrit, % RBC | 42.2 ± 0.9 | .01 | —.14 | —.26 | — | — |
| Liver GSH, mg/gm | 2.27 ± 0.16 | .04 | .28* | — | — | — |
| Weight, lbs. | 105.9 ± 2.5 | .54** | — | — | — | — |
| Heart Girth, in. | 87.6 ± 0.9 | — | — | — | — | — |

* 5% significance level
** 1% significance level

relation between these variables prior to the feeding period. None of the other correlations involving blood fat are significant.

The only other significant correlations are those between liver GSH and body weight, and between body weight and heart girth. In view of the significant correlation between liver GSH and weight, a significant correlation between liver GSH and heart girth would be expected.

Mean changes, 95% confidence intervals and pertinent correlations are presented in Table 5. Initial weight had no influence on the magnitude of change in any of the variables or on economy of gain. The correlations between initial blood GSH and rate and economy of gain are in the same direction and of about the same magnitude as those observed in cattle by Kidwell *et al.* (*loc. cit.*). The correlation of initial blood fat with rate and economy of gain is similar to that for blood GSH. Bohman and Wade (1958) found a correlation between plasma fat and rate and economy of gain in steers of about the same size, but in the opposite direction, to those observed in this study.

*Ewe Experiment*

The mean weight, heart girth, blood GSH and hematocrit, with 95% confidence intervals and the six correlations among them as determined prior to the temperature treatments are presented in Table 6. Comparison with the mean values observed on the lambs indicates no age effect on hematocrit or blood GSH.

Reid, Ward and Salsbury (1948) found a gradual increase in bovine blood GSH with increasing age. The correlation between blood GSH and hematocrit is similar to that observed with the lambs. The significant correlation of blood GSH with heart girth but not with body weight is in agreement with the findings of Kidwell *et al.* (1955) with beef cattle, but not with the lambs.

Analysis of variance indicated, as expected, no differences in blood GSH or hematocrit among the four groups prior to the temperature treatments. There was no evidence of any treatment effects. It must be concluded that under the conditions of this experiment temperature changes do not influence blood GSH or hematocrit in aged ewes. This is in agreement with the findings of Blincoe and Brody (1951), with respect to hematocrit. There was a significant decrease, independent of treatment effects, in hematocrit ($2.2 \pm 1.8$) and in blood GSH ($1.6 \pm .3$) between the first and second bleeding, but no change between the second and third bleedings. There is no immediately apparent explanation.

TABLE 5

Mean changes and feed economy during 60 day period with 95% confidence interval and pertinent correlations.

| Item | Mean and 95% Interval | Product-Moment Correlations | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Initial | | | | Final | | | | Change | | | |
| | | GSH | Hemato-crit | Blood fat | Weight | GSH | Hemato-crit | Blood fat | Weight | GSH | Blood fat | Hemato-crit | Weight (gain) |
| Change in blood GSH, mg % | 1.7 ± 1.6 | —.33* | —.41** | —.27 | .26 | .78** | .30* | —.03 | .20 | — | — | — | — |
| Change in blood fat, mg % | 20.8 ± 10.2 | —.40** | —.25 | —.50** | .02 | —.28* | —.28* | —.04 | .21 | .11 | — | — | — |
| Change in Hematocrit % RBC | 2.4 ± 0.9 | —.30* | —.47** | —.14 | .21 | .08 | .69** | —.17 | —.03 | .57** | .10 | — | — |
| Gain, lbs. | 18.5 ± 1.6 | —.02 | —.02 | —.31* | .01 | —.13 | —.31* | .06 | .69** | .05 | .22 | —.24 | — |
| Lb. feed/lb. gain | 13.4 ± 1.6 | .48* | .06 | .42* | .09 | .49* | .28 | .23 | —.58* | —.11 | —.04 | .26 | —.85** |

* 5% level of significance
** 1% level of significance

TABLE 6
Mean, Weight, Heart Girth, Blood GSH and Hematocrit with 95% Confidence Intervals and Correlations Among them as Determined Prior to Temperature Treatments.

| Item | Means | Product Moment Correlations | | |
|---|---|---|---|---|
| | | Heart Girth | GSH | Hematocrit |
| Weight, lbs. | 132.5 ± 4.3 | .79** | .18 | .38 |
| Heart Girth, in. | 36.7 ± .7 | — | .46* | .31 |
| GSH, mg % | 32.1 ± 3.5 | — | — | .48* |
| Hematocrit, % RBC | 41.2 ± 1.8 | — | — | — |

* 5% level of significance
** 1% level of significance

## Summary and Conclusions

Two experiments were conducted in an investigation of blood GSH levels in sheep. Hematocrit, blood fat and liver GSH were also measured. In the first experiment 48 lambs were assigned treatments for a 60 day feeding period in a 2 × 2 × 3 factorial design to determine main effects and interactions of two levels of chlortetracycline, two levels of stilbestrol and three levels of animal fat on blood GSH, hemoglobin, hematocrit, liver GSH, weight, rate and economy of gain. Relations and changes among the variables were also studied. The analyses indicate that under the conditions of this experiment the following conclusions are warranted.

1. Analysis of variance indicated no difference among the means of the treatment groups.

2. Blood GSH, total cell, hemoglobin, hematocrit, and fat levels of sheep are of similar magnitude to those in cattle.

3. Blood GSH, fat and hematocrit are not stable in sheep, but may change markedly. This is in agreement with observations in cattle. No explanation is immediately evident.

4. Blood GSH and fat were significantly correlated with subsequent economy of gain. Blood fat was associated with rate of gain.

5. There was no association between liver GSH and blood GSH.

The second experiment was conducted to determine the effect of ambient temperature on blood GSH level of the ewe. No temperature effects were observed. Age had no effect on blood GSH or hematocrit level (ewes vs. lambs).

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# THE EFFECTS OF PARATHYROID EXTRACT UPON THE TELEOST FISH, *Lebistes reticulatus*[1]

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## INTRODUCTION

ALTHOUGH there was early uncertainty concerning the endocrine nature of the parathyroid glands, once this was established it was found that their function was necessary for the maintenance of mineral balance and for the survival of animals having high calcium and phosphorus supplies. The presence of the parathyroid has been demonstrated for representatives of all vertebrate groups except fishes and certain amphibians which never undergo a true metamorphosis.

Manifestations of disorders due to either over- or under-activity of the parathyroid glands in the human have been observed and studied in many clinical cases, and experiments designed to simulate such conditions have been carried out on animals in the laboratory. The first emphasis was on the hypoparathyroid state as produced by partial or complete parathyroidectomy, but the preparation of effective parathyroid extracts by Collip and by Hanson independently in 1925 provided a tool for experimental study of hyperparathyroidism.

Most of the work has been concerned with mammals. Parathyroidectomy has been performed on rats, rabbits, guinea pigs and many other mammals, and has also been carried out in some amphibians, as *Rana catesbiana* (Waggener, 1930) and *Salamandra atra* and *Salamandra maculosa* (Scholz, 1935).

Albright and Reifenstein (1948) have fully reviewed the subject of bone diseases in humans with particular emphasis on studies involving hyperparathyroidism. With the use of parathyroid extract, hyperparathyroidism has been investigated in rats (Bauer, Aub and Al-

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bright, 1929; McJunkin, Tweedy and Breuhaus, 1932; Selye, 1932a; Pugsley and Selye, 1933; Burrows, 1938; de Robertis, 1940; Heller, McLean and Bloom, 1950), cats (Bauer, Aub and Albright, 1929), dogs (Hueper, 1927; Thomson and Pugsley, 1932), kittens, puppies and guinea pigs (Heller, McLean and Bloom, 1950). The first successful production of *osteitis fibrosa*, the typical bone disease of hyperparathyroidism, in laboratory subjects was reported by Jaffe, Bodansky and Blair (1930) as the result of their work carried out on guinea pigs and is analyzed in a series of papers (1930, 1931, 1932). Waggener (1930) studied the effects of parathyroid extract on anurans.

No work has as yet demonstrated any effect of parathyroid extract on those animals in which a parathyroid gland seems to be absent. Yet, if the parathyroid gland regulates calcium and phosphorus metabolism in other bony animals, it seems likely that there must be some tissue or organ performing a similar role in bony fishes and non-metamorphosing amphibians.

Recently Rasquin and Rosenbloom (1954), in a study of specimens of the teleost, *Astyanax mexicanus* (Filippi), maintained in darkness for long periods of time, found that the calcium supply was slowly depleted in the bones in a manner similar to that seen in hyperparathyroidism in mammals. They correlated with this the fact that the ultimobranchial (postbranchial) bodies of these animals exhibited hyperplasia and hypertrophy, and suggested that the ultimobranchial body may have a function similar to that of the parathyroid gland.

The present investigation is a study of the effects of administration of mammalian parathyroid extract at various dosage levels to specimens of the guppy (*Lebistes reticulatus*) to ascertain whether such treatment produces any changes in growth, in bone or kidney structure, or in the size of the ultimobranchial body.

## Materials and Methods

Adult specimens of the guppy, *Lebistes reticulatus*, were used in this experiment. Both males and females were treated, but the females predominated since males, because of their small size, were difficult to inject successfully. A total of 59 females and 25 males was treated with parathyroid extract and 25 females and 8 males served as controls. The length of each animal was determined at the beginning and at the end of the experiment; this, rather than the age of the fish, was used in setting up the series of controls for the treated animals. The

measurement was taken from the mouth to the emergence of the dorsal fin; this measurement ranged from 9.0 mm. to 21.0 mm. in females and from 9.0 mm. to 13.0 mm. in males. Live weights of the animals in water were also taken: the average weight for the females was 0.6 gms. and for the males, 0.2 gms.

Intraperitoneal injections of "Paroidin" (Parke, Davis & Co.) were administered to the fish in 0.01 cc. doses. Two concentrations (0.5 unit and 1.0 unit per injection) were given to separate groups. Injections were made at three-day intervals and the total number of injections varied from one to twenty over periods of one to sixty days. The controls received corresponding numbers of injections of distilled water. All animals were anesthetized before injection in a 1:6000 solution of tricaine methanesulfonate (MS 222).

Treated animals and controls were preserved for sectioning at various intervals after the beginning of treatment. Any fish which succumbed during the treatment were fixed as soon as possible after death. Fixation was in Bouin's fluid. Serial sections of the entire head and trunk were cut at 7 microns and stained with Mallory's Triple Stain.

Detailed comparisons were made between control and experimental animals with respect to the histological structure of skull bones, vertebrae and pharyngeal teeth. The kidneys and the ultimobranchial body were also studied histologically.

## Observations

*General Observations during Treatment.* Upon injection of parathyroid extract (PTH) there was an immediate response in the integument of many fish. The entire side of the body on which the injection was administered would frequently turn dark for several hours, later return to normal, and then become almost transparent for several days. Over the period of treatment, the parathyroid-injected fish showed an increase in body length in only two or three animals while all control animals showed an increase of 0.5 to 2.0 mm., depending on the length of the experiment. Many of the treated animals exhibited an actual decrease in body length ranging from 0.25 mm. to 3.0 mm., and a few had arched backs apparently resulting from some bone resorption in the vertebral column. A number of the females in both the treated and control groups gave birth to litters of young, but many of the young of the PTH-injected females were born pre-

maturely and soon died. About nine out of every ten of the young from the water-injected females lived to maturity as compared with only about three out of every ten from the parathyroid-injected females. This probably indicates that the parathyroid extract has an effect on embryonic development, but this has not been studied in the present work.

*Changes in Bone and Teeth.* Fish given the highest dosages (15-20 units of Paroidin over periods of 45-60 days) showed clear evidence of an increase in osseous tissue.

All specimens used were sexually mature but it must be remembered that the teleost skeleton, unlike that of mammals, maintains a steady, though slow, growth throughout life. For this reason the histological appearance of the bones of the skull in the fishes is more similar to that of young mammals than of adult mammals. Sections through several selected bones of the skull of control specimens serve to illustrate this. Figure 1 shows the maxillary bone applied to the quadrate cartilage at the region of the rostro-palatine articulation. The bone presents a lamellar appearance and is surrounded by a continuous layer of osteoblasts. Osteocytes within the bone are quite sparse and no definite periosteal layer is to be seen. All of these features are characteristic of actively growing bone. A similar situation exists in most other bones of the skull including those formed by intracartilaginous ossification (Figure 2). A possible exception may be noted in the suborbital bones which form thin platelets encircling the suborbital lateral line canal (Figure 3). These do not appear to retain a continuous layer of osteoblasts at the periphery and do seem to have a thin but definitely established periosteal covering.

Figures 4, 5, 6 illustrate the changes seen in the corresponding skull bones of fishes which had received long treatment with parathyroid extract. The bones are thicker and appear to be denser so that they stain more heavily. Osteoblasts are present around the periphery of the bone in even greater number than in controls, but fibroblast-like cells in the region surrounding the bone are also more numerous than in the controls. No increase in osteocytes can be demonstrated and no osteoclasts can be identified with certainty. The increase in the thickness and density occurs fairly uniformly in all skull bones including the suborbitals which, on the basis of their appearance in controls, might have been expected to show a somewhat different response

Study of the vertebral column at four specific levels showed that

similar changes occurred here. Comparison between control and experimental animals in this region is illustrated in Figures 7, 8, 9, and 10. Ossification of the vertebral centra and of the neural arches is more extensive in experimentals than it is in controls in every case.

Some attention was also given to the histology of the pharyngeal teeth. These are very numerous in *Lebistes* and, in a single section, may often be observed in all stages of development from small, deeply embedded tooth germs to fully erupted functioning teeth. In experimental animals the teeth proved to be larger particularly at their bases, and more densely stained than in the controls (Figures 11 and 12).

Although animals receiving the highest dosage exhibited these indications of greater calcification and ossification throughout the skeletal system, those given a smaller number of injections presented a quite different appearance. Fish given only a very few injections (1 to 3 injections of either 0.5 unit or 1.0 unit) showed no discernible histological differences in skeletal structure from that of controls. However those which received 4 to 8 injections of 1.0 unit each or 7 to 14 injections of 0.5 unit each exhibited a decrease in ossified tissue as compared with controls. This is illustrated in Figures 13, 14, 15, and 16 which show a contrast between the size and density of bone in several regions of the skull and vertebral column. Examination of the bone under the oil immersion lens revealed that the usual continuous layer of osteoblasts is not present in the experimental animals but that, instead, fibroblasts predominate in immediate contact with the bone surface. The indication is that formed bone is actually being destroyed in these animals, but no cells definitely identifiable as osteoclasts could be distinguished.

Fish given dosages of 9 to 14 injections of 1.0 unit each or 15 to 20 injections of 0.5 unit each either showed no well-defined difference in bone structure between control and experimental animals or a slight increase in bone deposition in the treated specimens.

These observations indicate that the parathyroid treatment does effect changes in the skeletal system of *Lebistes* and that the response elicited by short term treatment is quite different from that seen in prolonged treatment. A short course of injections results in destruction or resorption of bone but long-continued treatment causes excessive bone deposition.

*Kidney.* The kidneys were examined in detail in all specimens since

## PLATE I

*All figures are unretouched photomicrographs*

All sections figured are of *Lebistes reticulatus,* cut at 7 microns, and stained with Mallory's Triple Stain.

Figure 1. Cross-section of the head region of a control animal showing the maxillary bone (MB) closely applied to the quadrate cartilage (QC). 275 X.

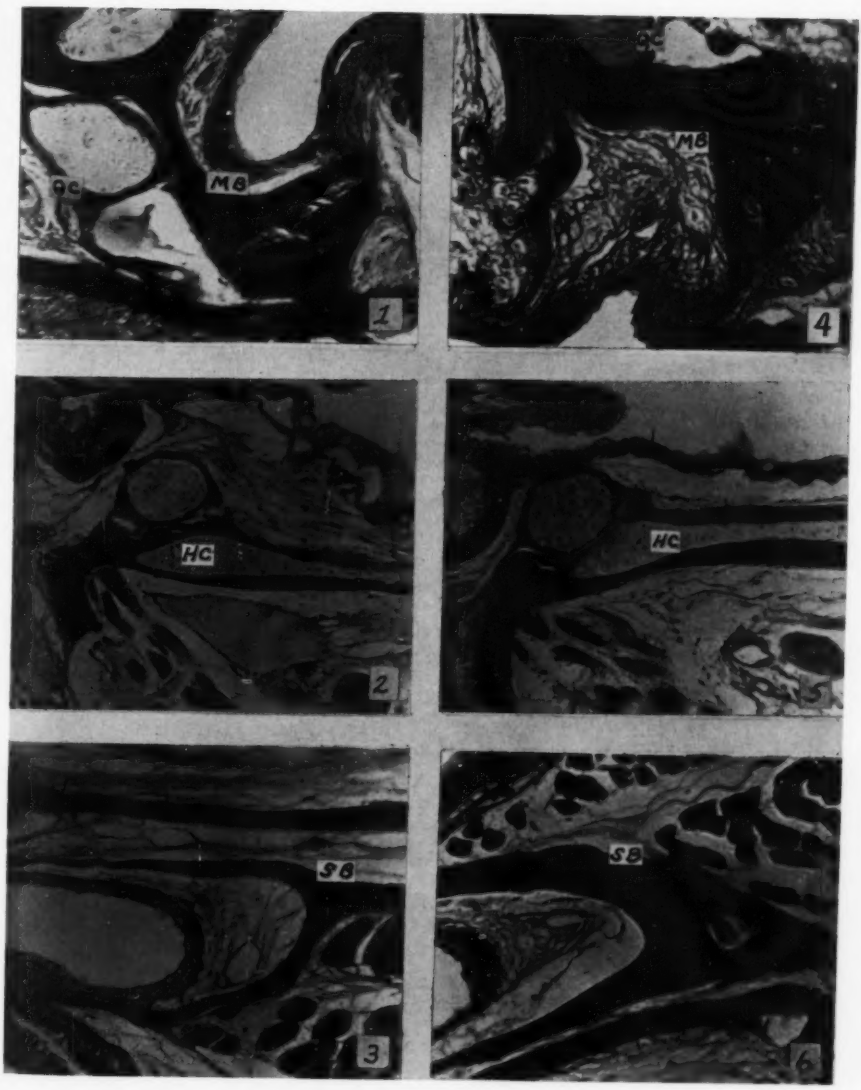
Figure 2. Cross-section through the skull of a control animal illustrating intracartilaginous ossification. The hyaline cartilage (HC) serves as the model on which the bone is laid. 275 X.

Figure 3. Cross-section through the suborbital bone (SB) encircling the suborbital lateral line canal in a control animal. 275 X.

Figure 4. Cross-section through the head region of an animal treated with 20 injections of 1.0 unit of parathyroid extract. Greater bone deposition has occurred in this animal than in the control of figure 1. 275 X.

Figure 5. Cross-section through the skull of an animal treated with 20 injections of 1.0 unit of parathyroid extract. A thicker border of bone surrounds the cartilage than in the control of figure 2. 275 X.

Figure 6. Cross-section through the suborbital bone (SB) encircling the suborbital lateral line canal in an animal treated with 20 injections of 1.0 unit of parathyroid extract. The suborbital bone has increased in thickness and density to a greater extent than its control of figure 3. 275 X.


MB
OC
1
OC
MB
4
HC
2
HC
5
SB
3
SB
6

## PLATE II

*All figures are unretouched photomicrographs*

Figure 7. Cross-section through vertebral column of a control animal showing the vertebral centrum and a portion of the neural arch. 275 X.

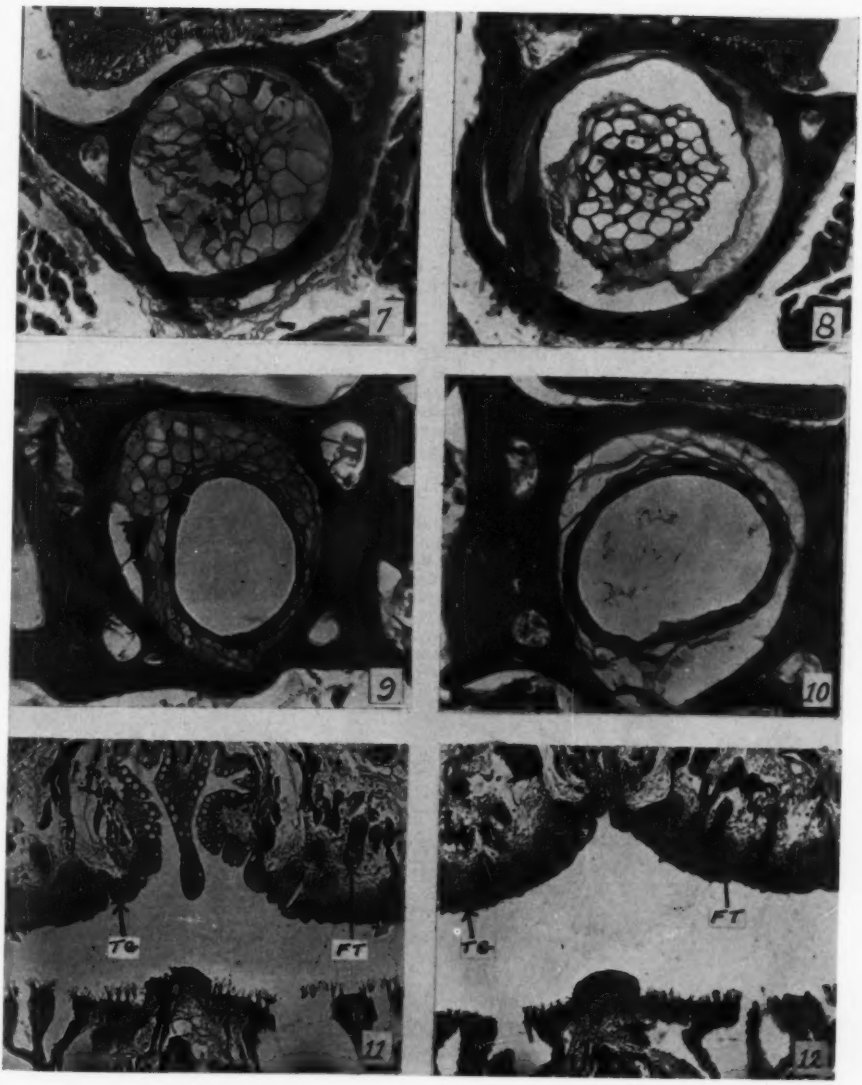
Figure 8. Cross-section through vertebral column of an animal treated with 20 injections of 1.0 unit of parathyroid extract. This illustrates the greater bone deposition in the experimental animal at this stage than in the comparable control of figure 7. 275 X.

Figure 9. Cross-section through vertebral column near the end of the body cavity in a control animal. 275 X.

Figure 10. Cross-section through vertebral column near the end of the body cavity in an animal treated with 20 injections of 1.0 unit of parathyroid extract. Bone deposition is more advanced in the region of the vertebral centrum here than in the control of figure 9. 275 X.

Figure 11. Cross-section through the pharyngeal area of a control animal exhibiting the functioning teeth (FT) and the embedded tooth germs (TG). 100 X.

Figure 12. Cross-section through the pharyngeal area of an animal treated with 20 injections of 1.0 unit of parathyroid extract. Both the functioning teeth and the tooth germs have undergone slightly greater development than in the corresponding control of figure 11. 100 X.


7
8
9
10
TC
FT
11
TC
FT
12

## PLATE III

*All figures are unretouched photomicrographs*

Figure 13. Cross-section through the head region of a control animal showing the maxillary (MB) bone closely applied to the quadrate cartilage (QC). 275 X.

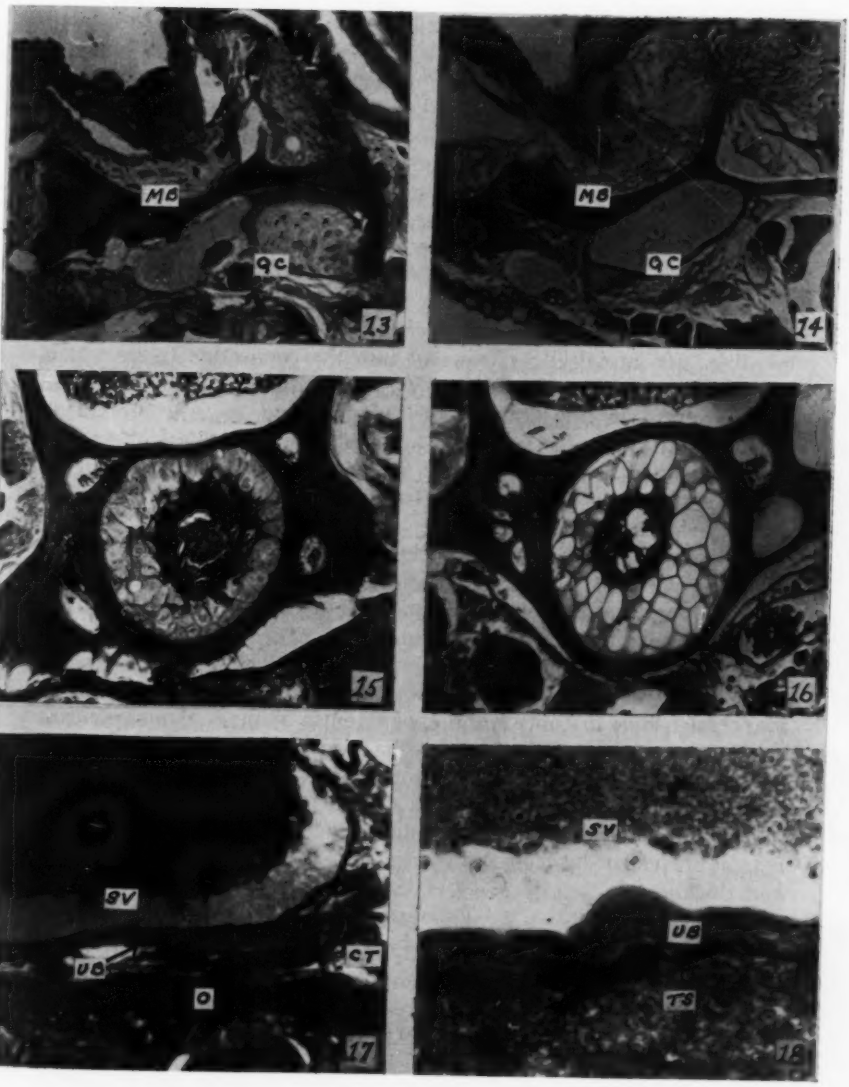
Figure 14. Cross-section through the head region of an animal treated with 7 injections of 1.0 unit of parathyroid extract. A slight decrease in ossified tissue is noted when compared to the control of figure 13. 275 X.

Figure 15. Cross-section through the vertebral column near the end of the body cavity in a control animal. 275 X.

Figure 16. Cross-section through the vertebral column near the end of the body cavity in an animal treated with 13 injections of 0.5 unit of parathyroid extract. The treated animal shows less deposition of bone as compared to that of the control in figure 15. 275 X.

Figure 17. Longitudinal section of a control animal showing the thin attenuated ultimobranchial body (UB) between the sinus venosus (SV) and the oesophagus (O). The lateral end pushes into the connective tissue (CT) lining the oesophagus. 275 X.

Figure 18. Cross-section of the ultimobranchial body (UB) of a control animal. Its few layers of cuboidal cells with prominent nuclei stretch across the mid-line of the body anterior to the transverse septum (TS) and beneath the sinus venosus (SV). 600 X.


MG
QC
13
MG
QC
14
15
16
SV
UB
CT
O
17
SV
UB
TS
18

metaplastic ossification in the kidneys is frequently observed in hyperparathyroidism in mammals. No changes were found in *Lebistes*.

*Ultimobranchial Body*. In view of the observations of Rasquin and Rosenbloom (1954) which indicate a possible parathyroid-like function for the ultimobranchial body in fishes, a special study of the ultimobranchial body of the control and experimental *Lebistes* was undertaken. Unfortunately the ultimobranchial body of this fish is extremely thin and attenuated (Figures 17 and 18). It is therefore much less favorable for study than is the rounded compact ultimobranchial body found in many teleosts. In *Lebistes*, the body lies anterior to the transverse septum in close contact with the sinus venosus. At most levels it is only a single layer of cells but it expands slightly in the mid-line. Its lateral edges fray out into the connective tissue which lies just beneath the oesophagus. The cells making up the body are quite uniform in appearance, are cuboidal and have a prominent nucleus filling over half the cell. The nucleus is elliptical with a sharply defined nucleolus. In this body, no lumina corresponding to those described by Krawarik (1936) and Eggert (1938) for certain other teleosts are discernible.

Comparison of the ultimobranchial bodies of control and treated fishes revealed no significant histological differences in either short term or long term experiments. Since the administration of parathyroid material might be expected to result in decreased activity and possibly in atrophy of any tissue which normally carries on a parathyroid-like function, careful measurements were made of the ultimobranchial bodies in control and experimental animals. No significant differences were found in average dimensions of the body in the two groups. It must be noted however that the shape of the ultimobranchial body in this species makes it difficult to estimate accurately its total volume and differences in volume could be present which might not be revealed by comparing measurements of maximum width and thickness.

## Discussion

It is well demonstrated that in mammals the parathyroid gland functions as a regulator of calcium and phosphorus supply in maintaining a mineral homeostasis necessary for the survival of the organism. The exact manner in which it fulfills this role is still uncertain. Selye (1942) has long held that its primary action is to promote mineral exchange between bone and blood. During development, bone may

be preceded by either cartilage or fibrous connective tissue. Intracartilaginous ossification begins with the hypertrophy and degeneration of hyaline cartilage. The adjacent matrix calcifies, the remaining cartilage is eroded away, and osteogenic cells, osteoblasts, are formed. The osteoblasts are separated from the cartilage matrix by a new thin layer which calcifies as it is being laid down; this is the bone being formed. However, bone is not an inert tissue and while the process of deposition is taking place, one of resorption is also occurring. Some of the osteoblasts fuse or are transformed into multinucleated cells, osteoclasts; these latter cells are presumably phagocytic in removing calcium and phosphorus from the bone with its resulting absorption. Ordinarily these processes are in balance and osseous tissue is constantly being renewed. Intramembranous ossification takes place through the hypertrophy of connective tissue cells and the transformation of their interstitial substances. The tissue becomes calcified and a continuous layer of osteoblasts appears on the surface of the developing bone; the osteoblasts aid in the formation of successive bone layers by their constantly serving as the basis for new tissue and by dividing off new osteoblasts on the bone surface.

According to Selye (1932b) and Thomson and Pugsley (1932) and others, hyperparathyroidism has direct effects on bone. In chronic oversecretion of the parathyroid hormone or after treatment with excessive dosage of parathyroid extract, a demineralization of bone occurs. Histologically this change is manifested by a greatly accelerated shift of osteoblasts to osteoclasts in the tissue. Much of the bone disappears and is replaced by fibrous tissue. Regions of most active bone formation are reported to be most susceptible to the action of the parathyroid hormone. In some cases, metastatic bone and calcium deposits may be found in soft tissues such as kidney, heart muscle and intestinal walls.

Albright (1929) and his associates, on the other hand, maintain that the primary action of parathyroid hormone is on the kidney and results in an increased excretion of phosphorus. This causes a low phosphate and a high calcium level in the blood. The serum calcium level exceeds the renal threshold, and calcium is eliminated in the urine. When the system readjusts itself, the bones are attacked for replacement of the minerals and the histological changes in the bone which are described above then ensue.

Most of the work in experimental hyperparathyroidism has been

conducted on the mammal, and for this reason the results of the present investigation are compared with these studies. Yet it is not implied that what takes place in the teleost is necessarily governed by the same mechanism as that which obtains in the higher vertebrates. MacCullum and Voegthin (1909) claimed that treatment of parathyroidectomized patients with glandular material "even from an animal of widely different character" ameliorates the ill effects of the operation. This was considered to indicate a non-specificity of the parathyroid substance; nevertheless, subsequent work has demonstrated that the effects of parathyroid administration do differ markedly in different laboratory mammals, and it seems likely that animals belonging to different classes of vertebrates may be quite dissimilar in their mode of response to the hormone.

The dog, usually employed in assay methods, consistently exhibits the characteristic bone effects of hyperparathyroidism. With long continued injections these are manifested by decalcification and resorption of the existing bone, fibrous replacement of the marrow, and production of other symptons of *osteitis fibrosa*. The changes are generalized, but are most marked in the long tubular bones.

In immature rats, treatment with small doses of extract (1 to 5 units per day) causes a decrease in the amount of trabecular bone as the immediate reaction, but after treatment for a long period of time quite the reverse is true: there is first a tendency to return to normality and later an increase in the number of lamellae and trabeculae. Ultimately a condition of abnormal bone formation, known as "marble bone disease," ensues. A strong single injection in the young rat or in the puppy causes rapid resorption of bone and this is followed by slow regeneration of osseous tissue. In the adult rat, as in the growing animal, a prolonged treatment leads to the formation of huge bony deposits. There is no qualitative difference in the reaction of slow-growing and fast-growing bones but rapidly growing bones, as would be expected, are the first to show detectable effects.

The guinea pig which has probably been most used in parathyroid studies, differs markedly from the rat in its responses. It is generally agreed that there is little alteration after long treatment of the young with small injections. Jaffe, Bodansky and Blair (1932a) claimed that this is due to a "healing process" which has its beginning about 48 hours after the beginning of treatment: the immediate effects in long treatment are thus masked. Single injections do cause bone resorption

in the young guinea pig, but the dosage required is very high compared with that which produces similar effects in the dog. Administration of single large doses of parathyroid extract to adult guinea pigs causes no effects on bone but does result in a slight change in serum calcium after 24 hours.

In kittens and cats, there seems to be a sustained resistance to parathyroid extract. No matter what the method or the size of the injections, these animals suffer little, if any, bone damage as the result either of prolonged treatment or of single toxic injections. The rabbit also shows little change after lengthy treatment, but some workers claim that rapid and extensive decalcification occurs in rabbits given a large dose of parathyroid extract.

In Waggener's (1930) study of the effects of parathyroid administration in the frog a hypercalcemia was noted which may perhaps be interpreted as resulting from calcium withdrawal from the bone. Unfortunately, however, no study of the histology of bone was undertaken in Waggener's investigations.

In the experiment here reported, the fact that there were changes in the bone as a result of the injection of parathyroid material indicates that the fish reacts to parathyroid treatment, even though no functional gland comparable to that of vertebrates has as yet been described for fishes. The changing pattern of effects with short-term and long-term treatment found in these experiments parallels fairly closely that described for the growing rat (Bauer, Aub and Albright, 1929; Burrows, 1938). The dosage levels used in the present study must be considered as quite high, the amount of "Paroidin" injected per dose being higher in comparison to the weight of the experimental animal than that used in most studies on mammals. As has been pointed out earlier, although the fish used were sexually mature, they were still exhibiting a steady increase in total length and a corresponding steady skeletal growth. The processes occurring in the bone may therefore be considered as corresponding to those found in immature rats and it appears that the effects of parathyroid administration upon these processes are similar in the two animals. Short term treatment with high doses causes bone destruction while long term treatment causes excessive bone deposition. It is also to be noted that in the rat, as in the fish, no metastatic calcification in soft tissues occur as a result of parathyroid treatment (McJunkin, Tweedy and Breuhaus, 1932).

Waggener (1930) found that single large injections (80 units) of parathyroid extract did not prove fatal to frogs and suggested that amphibians may have a very high resistance to "parathyroid poisoning." It is doubtful whether this is true for the fish. Many of the experimental animals died after several injections. In some cases this may have been due to injury during the injection but, since relatively few of the control animals succumbed even though they received injections of the same volume, it does appear that the parathyroid extract did often have toxic effects.

In discussing the possibility that certain bones, or certain parts of bones, respond to PTH more readily than others, Bauer, Aub and Albright (1929) suggested that the "trabeculae seemingly served as the labile supply of calcium and the cortex as the stable supply." This theory was criticized by Jaffe, Bodansky and Blair (1931b) who held that all osseous areas are subject to the resorption-deposition effect of the parathyroid hormone, but because of the fact that some bones are more active in their growth processes than others, the response appears more acutely in the metabolically more active regions. This allows for changes both in the cortices and the trabeculae depending upon the growth rates of these parts at the time of treatment. Burrows (1938) further elaborated this idea in relation to slow-growing and fast-growing bones, the decalcification or hypercalcification, occurring only slightly earlier in the latter group. For these reasons the classification of bones according to their response to PTH as based on their rate of growth would cease to be applicable in the adult and there would be a tendency toward uniformity in the reaction (Jaffe, Bodansky and Blair, 1931b). In the fish used in these present experiments, the reactions to PTH administration were generalized; all bones studied showed the same type of response to a given dosage of the hormone.

In addition to the effects on osseous tissue, parathyroid treatment also resulted in a cessation of growth in nearly all of the experimental fishes and an actual shorting of the body in some. Decrease in stature resulting from hyperparathyroidism has been reported for the human (Selye, 1947) and also occurs in experimentally treated rats (Robinson and Thompson, 1932; Burrows, 1938), dogs (Jaffe and Bodansky, 1930), rabbits (Robinson and Thompson, 1932), and axolotls (Thompson and Huxley, 1934). This was at first considered to be attributable solely to fractures and bone degeneration in the vertebral column but

such changes, while they do occur in some cases, are not invariably present. Robinson and Thompson (1932) proposed that the retardation of growth rate is due to an anti-growth factor produced by the parathyroid gland. The work of Thompson and Huxley (1934) provides some support for this idea. They found that the axolotl, which apparently lacks a parathyroid gland and shows no changes in bone structure after parathyroid treatment, does respond by a marked retardation of growth rate which is directly proportional to the PTH dosage. It may well be that this factor is responsible for the same phenomenon in the fish treated with parathyroid extract.

Thompson and Huxley (1934) also noted a lightening in the color of axolotls given PTH injections. Smith and Hartman (1943) treated isolated fish scales from the goldfish (*Carassius auratus*) and the orange chromide (*Etroplus maculatus*) with solutions of parathyroid extract and obtained partial or complete contraction of melanophores within twenty minutes. They found the scales of *Lebistes* were too small and difficult to handle to obtain reliable results. The changes in coloration after injection in the present experiment may support the idea that some melanophore-affecting principle is present in parathyroid extracts.

Several factors have been considered operative in the amount of alteration caused by excessive PTH administration. The age element has been studied in several experiments of Jaffe, Bodansky and Blair (1930, 1932) whose investigation of the dog and guinea pig indicated an inverse correlation between age and damage to osseous tissue. The adult, as noted previously, always showed an absence of or a lesser response to parathyroid extract than the young of the same species where severe bone lesions always seemed to follow large single doses of the extract. This was attributed to a more rapid rate of mineral metabolism in young, actively growing animals; in addition it was suggested that an unidentified compensating mechanism may be present in adult animals which is absent or less effective in the young. In the present study, the fish used were all of approximately the same age. The relation between age and sensitivity to PTH administration in fishes remains for future investigation.

All mammalian studies indicate that there is no relation between sex and response to PTH treatment. This seems to be true for *Lebistes* also.

The question of species-specificity has already been touched upon.

Jaffe, Bodansky and Blair (1930) found that very different dosages of PTH were required to bring about comparable bone changes in dogs and guinea pigs while rabbits and cats seemed almost completely immune to the treatment. The number of studies concerning lower vertebrates is so small that nothing can be said concerning species-specificity in these forms.

Rasquin and Rosenbloom (1954) raised specimens of *Astyanax mexicanus* (Filippi) in total darkness for periods of several months to two years and found many abnormalities in various organ systems indicating serious endocrine imbalances. Among other organs affected, the kidneys were found to have distended tubules, calcium deposits, and papillary adenomas. Effects on bone involving loss of calcium and fibrosis of the vertebral arches were also obtained. The ultimobranchial bodies of these fish were found to be hypertrophied. These investigators suggested that these findings may indicate that the ultimobranchial bodies have a parathyroid-like function. The hypertrophied bodies were considered to be hyperactive, producing a condition comparable to hyperparathyroidism, the kidney and bone effects being then attributable to this condition. Whether or not this hypothesis is correct must be ascertained through further experimentation. It may be suggested that the bone changes observed could have resulted from a deficiency of vitamin D and that the hypertrophy of the ultimobranchial body could have resulted from the general endocrine imbalance which occurred. It is known that the ultimobranchial body can be stimulated to hypertrophy by injections of thyrotropic hormone (Eggert, 1938) and it may be that abnormally high levels of other hormones may also produce this result.

The morphology of the ultimobranchial body in a large number of teleosts has been studied by Krawarik (1936). A wide variety of morphological types exists. Some fishes have rounded, compact, paired bodies; others have unpaired, sheet-like bodies; and a number of intermediate types may be distinguished. Unfortunately, *Lebistes* proved to have an extremely thin, attenuated ultimobranchial body resembling most closely that described for *Perca fluviatilis* L. by Krawarik. Comparison of these bodies in the control and experimental fishes used in the present work showed no discernible differences. It is clear, however, that because of the shape of the structure, volume differences would be difficult to detect unless they were quite extreme. These experiments, therefore, furnish no reliable evidence concerning

the possible parathyroid-like function of the ultimobranchial body in fishes.

## Summary

1. Fish of the species, *Lebistes reticulatus,* were injected with parathyroid extract dosages of 0.5 unit and 1.0 unit given at three-day intervals for various periods of time.

2. Bone changes were studied and differences in response were correlated with the length of treatment. No changes in osseous tissue occurred in animals receiving only a small number of injections, those receiving a somewhat large number showed bone resorption, those receiving the highest number had excessive deposition.

3. These results resemble the changes after similar treatment in immature rats.

4. No calcification or bone deposition occurred in the kidneys.

5. Definite effects of the treatment upon the ultimobranchial body were not discernible but, because of the shape and size of the body in *Lebistes,* this result is not considered conclusive for the group.

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# THE METABOLIC EFFECT OF CORTISONE ACETATE DURING SALAMANDER LIMB REGENERATION

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## INTRODUCTION

THE adreno-cortico steriods, particularly cortisone or Compound E, are known to be growth inhibitors of adult tissues. This inhibition has been explained by assigning to these hormones an action which either hinders the anabolism of proteins (Reifenstein, Albright, and Wells, 1945) or enhances their catabolism (Long, Katzin, and Fry, 1940). In an earlier paper (Manner, 1955) these metabolic concepts were strengthened by demonstrating that cortisone inhibited the speed of the wound healing process by retarding mitotic activity. Although many tissues exhibit these cortisone-induced inhibitory effects, the epidermis and connective tissues appear to be most drastically affected.

During regeneration of the adult urodele limb, an embryonic blastema forms at the amputation surface. This blastema is essentially a mound of pleuripotential embryonic connective tissue cells, the fibroblasts (Manner, 1953) covered with new epidermis produced by the region contiguous to the amputation surface. It was felt that this blastema would serve as an ideal site for testing the metabolic effects of cortisone on *embryonic* epidermis and connective tissues.

In the epidermis of an intact adult salamander limb, there is no appreciable growth or degeneration. Protoplasm is continually being produced, however, to replace those cells normally lost in periodic moulting. This appears to indicate that a state of dynamic equilibrium exists between the anabolic and catabolic phases of protein metabolism. In a regenerating limb this metabolic equilibrium is destroyed, for in addition to the replacement activity, new growth occurs at the amputation surface. This necessitates an increase in the protein anabolism. If the metabolic effects of cortisone are as pronounced in embryonic tissues as they are in the adult, either anabolism should be reduced or catabolism enhanced. In either case, an inhibition in the

rate of regeneration should be evidenced. The experiments reported in this paper attempt to test this hypothesis.

## Materials and Methods

The adult form of the newt, *Trituris viridescens viridescens* (Rafinesque) was selected for this study. Uniformity of size was attained by rejecting all animals whose nose-anal length was greater than 145 mm. or less than 138 mm. The average weight of those selected, calculated by water displacement, was 2.4 grams. A total of 116 animals was used. These were separated into an experimental group of 80 and a control series of 36. All of the salamanders were maintained at a laboratory temperature of 19 ± 1° C. and were fed twice weekly on live *Tubifex* worms.

The right forelimb of each animal was amputated through the radius and ulna at a point equidistant from the wrist and elbow. After amputation each experimental animal received daily intramuscular injections of 0.037 mgm. cortisone acetate while the controls were given daily injections of Holtfreter's solution. In addition to recording the gross morphological changes during a 35-day regenerative period, the limbs of selected animals were prepared for histological examination.

## Experimental Observations

### *A. Controls*

Within 24 hours after amputation, the epidermal cells from the stump migrated over the cut surface and closed the wound. There were no signs of inflammation or degeneration. After 18 post-operative days the blastema was well formed at the amputation surface (Fig. 3). This blastema continued to grow throughout the 35-day period. The external appearance of the paddle stage at 30 days was evidence of a normally progressing internal differentiation. Throughout the 35-day regenerative period, the animals appeared healthy and no deaths occurred.

### *B. Cortisone Treated Animals*

The entire wound healing process in those animals receiving the daily injections of cortisone acetate was abnormal in many respects. The initial observation was a delay in the primary wound closure. This normally occurs within 24 hours after amputation. In these animals it took a minimum of 48 hours. The limb never did lose its

inflamed appearance and at 6 days, when a blastema normally begins to form, these limbs had no such structure (Fig. 6). At 7 days the wound reopened revealing the cut ends of the radius and ulna. The subsequent changes were rapid. Following the reopening of the wound a continuous degeneration of all the tissues of the limb began in the amputation area and proceeded in a disto-proximal direction. This extensive degeneration involved the epidermis, dermis, muscle, connective tissues, and nerves. The only limb elements which remained after 18 post-amputation days were the radius and ulna. These remained attached to the humerus by tendon remnants (Figs. 4 and 5).

A histological examination revealed this same cytolytic picture. In addition to large numbers of phagocytic white blood cells which had invaded the connective tissues (Fig. 9), osteoclasts were found in pits in the bone (Fig. 8) indicating that even bone was being destroyed. The presence of large numbers of phagocytes is probably due to the leucotaxic effect of the degenerating tissues (Menkin, 1940).

## Discussion

The overall appearance of this regeneration syndrome is strikingly similar to that observed by others after transection of the brachial nerves (Butler, 1949, Butler and Schotte, 1941, Singer, 1946). The conclusion drawn by these workers indicated that nerves are necessary for halting the process of degeneration or dedifferentiation and initiating the process of blastema formation. In the present work it would appear that an excessive amount of cortisone has the same general effect as the removal of the nerves from the regenerating limb. This suggests that the effects of the brachial nerves and cortisone in regeneration are opposite. As both of these factors are present normally in the salamander limb, the effect of each might be to check the action of the other. This hypothesis is readily testable.

Although the work of Butler (1933, 1935) leaves little doubt that the cellular elements of the blastema are of local origin, it appears that the control of the regenerative process is systemic, perhaps of a nervous and hormonal nature. The metabolic depressing effect of cortisone acetate has been previously demonstrated and it would appear that the present work substantiates these findings.

FIGURE 1
The normal unamputated right forelimb

FIGURE 2
The right forelimb immediately after amputation

FIGURE 3
The control limb 18 days after amputation. A well formed blastema is present at the amputation surface.

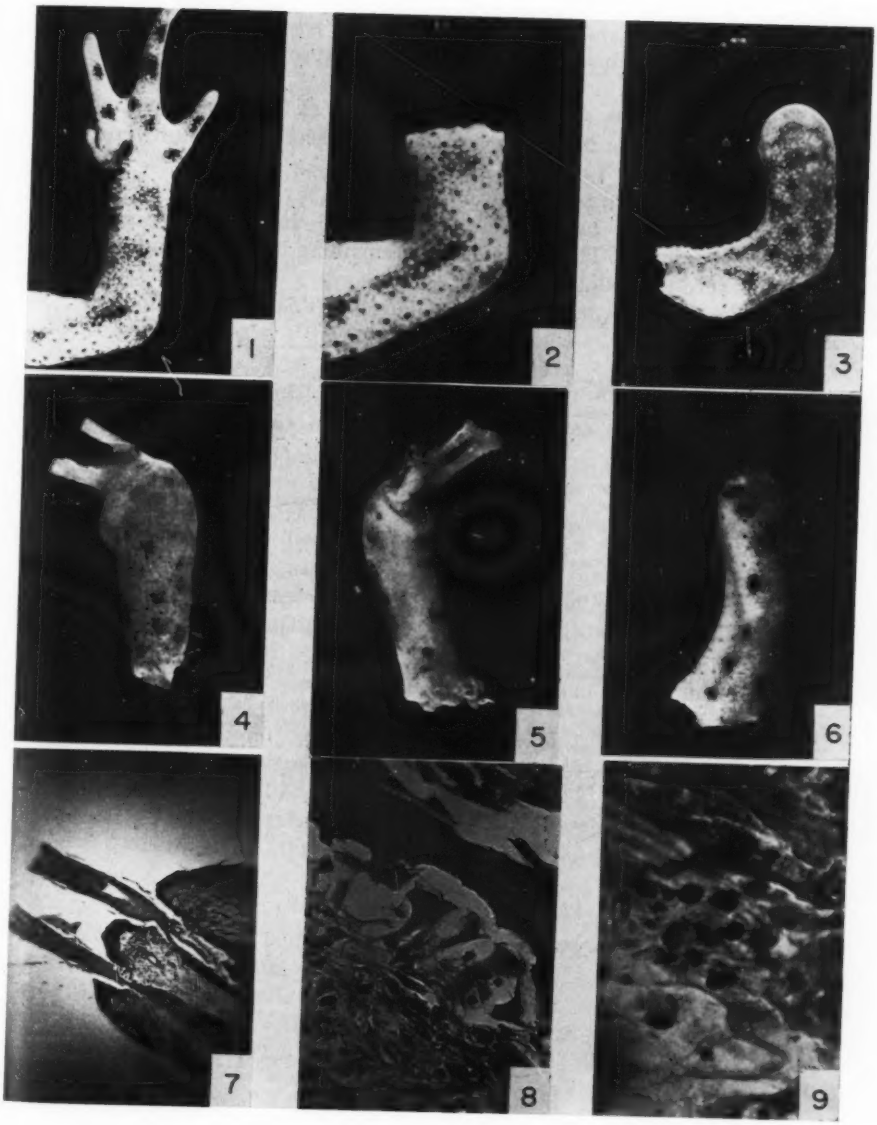
FIGURES 4 and 5
The limbs of cortisone treated animals 18 days after amputation. Note the protrusion of both the radius and ulna.

FIGURE 6
Limb of a cortisone treated animal 6 days after amputation. The close proximity of the wound healing epidermis to the underlying radius and ulna indicates the lack of any formative blastema.

FIGURE 7
Low power through the distal end of the limb of a cortisone treated animal 18 days after amputation.

FIGURE 8
High power through the same section depicted in figure 7. The multinuclear giant cells can be seen in the bone pocket.

FIGURE 9
Section through the limb of a cortisone treated animal 18 days after amputation. The connective tissue contains many polymorphonuclear leucocytes.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## Summary

1. The right forelimbs of 116 salamanders were amputated through the radius and ulna.

2. Eighty animals received daily injections of 0.037 mg. cortisone acetate. The control animals received daily injections of Holtfreter's solution.

3. Regeneration was normal in the controls.

4. Degeneration, rather than regeneration, characterized the experimental animals. The only elements remaining in the forelimb after 18 days were the radius and ulna.

5. The possible mechanisms underlying this degeneration are discussed.

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# CYANIDE ADAPTATION IN *CHILOMONAS PARAMECIUM* AS RELATED TO THE TYPE OF AVAILABLE NUTRIENT[1]

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## Introduction

It has been demonstrated that the ciliate, *Tetrahymena pyriformis* W may, upon continuous exposure to potassium cyanide, become increasingly resistant to cyanide inhibition of growth and respiration (14, 15). Evidence indicates that this resistance is due to physiological adaptive changes and not to selection of mutant forms either present initially or occurring spontaneously during the adaptive process.

Studies on the possibility of an adaptive phenomenon in Tetrahymena were initiated by the observation that cyanide, in low concentration, is stimulating both to growth and to respiration (16). Recently, Elrod (6) has reported that growth and respiration of the colorless flagellate, *Chilomonas paramecium*, is stimulated by low concentrations of cyanide, although higher concentrations are inhibitory. In view of these observations, it seemed logical to assume that Chilomonas also might possibly adapt itself against cyanide inhibition. In addition, the simplicity of the culture medium might prove valuable for future analysis of biochemical differences in cells under adaptive conditions. For these reasons the following tests have been conducted.

## Materials and Methods

Cells of a clone culture of *Chilomonas paramecium* were used in the study. All cultures were maintained in darkness at 25° C.

Two types of media were employed, both at pH 6.8. Initially the ammonium-acetate (AM-AC) medium of Pace (17), at a ten-fold increase in concentration, was used. Later tests were conducted with

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a medium containing 1 part of AM-AC with 4 parts of a one per cent aqueous solution of Proteose Peptone (Difco) and glucose. The latter medium will be referred to as "APG" and has the following composition:

| | | |
|---|---|---|
| Na-acetate | 84 | mgm |
| $NH_4Cl$ | 34 | mgm |
| $(NH_4)_2SO_4$ | 7.5 | mgm |
| $K_2HPO_4$ | 15 | mgm |
| $CaCl_2$ | 0.75 | mgm |
| $MgCl_2$ | 0.75 | mgm |
| Thiamin hydrochloride | 0.00075 | mgm |
| $FeCl_3$ | 0.0075 | mgm |
| Proteose peptone | 750 | mgm |
| Glucose | 750 | mgm |
| $H_2O$ (triple distilled) | 100 | ml |

Twenty ml. portions of appropriate medium were autoclaved for 15 minutes at 15 pounds steam pressure in 50 ml. pyrex erlemeyer flasks, the tops being covered with inverted 30 ml. beakers. The flasks were then sealed with sterile silicone stoppers (West Co., Inc., Phoenixville, Pa.). Series inoculations were made in such media at 3-day intervals with 0.5 ml. of parent culture. When transferred in this manner, cells were obtained in the logarithmic phase of growth and only a minimum of lag occurred in new cultures, thus assuring a high degree of physiological activity in the cultures.

Attempted adaptation to potassium cyanide was accomplished as has been previously described (14). Gradually increasing the concentration of KCN in serial transfers, cultures were finally maintained in $5 \times 10^{-6}$ M KCN or $10^{-5}$ M KCN in AM-AC or APG medium, respectively. Such cultures will be referred to as "adapted," it being assumed initially that a certain degree of resistance against cyanide might have been gained as a result of the prolonged exposure.

Previous to the "adaptation" to KCN in APG medium, it was actually necessary to adapt Chilomonas to the medium itself. Initially attempts were made to grow cells in medium containing only proteose peptone and glucose but without added acetate. However cells failed to grow in such medium. This is not surprising, since Holz (7) has shown that the flagellate is generally incapable of utilization of amino acids or glucose. Cells were finally caused to grow in APG medium as a result of a series of transfers into medium containing increasingly greater amounts of proteose peptone and glucose and decreasing amounts of ammonium salt and acetate.

Growth tests were conducted using ten flasks of medium for a particular condition. The number of cells per unit volume of a 3-day parent culture was determined by direct count of an aseptically withdrawn and properly-diluted alequote sample. Inoculation was accomplished by addition of such a quantity of the parent culture to obtain a cell concentration of 1000 cells per ml. of fresh experimental culture. In a given test 20 cultures were prepared of normal cells and the same number of cultures of "adapted" cells. To half of the cultures of each type sterile KCN was added in concentration ten times that used in stock cultures of the adapted cells. Thus for cells growing in AM-AC medium, the final test concentration was $5 \times 10^{-5}$ M KCN and in APG medium it was $10^{-4}$ M KCN. All cultures were incubated for 3 days in darkness at 25° C.

At the end of the growth period the optical density of all cultures was determined with a Coleman spectrophotometer at a wave length of 450 μ. All cultures in close agreement regarding optical density were pooled and the following tests conducted to evaluate the growth response.

The population density of the cultures was determined by direct count of a properly-diluted sample of the pooled cultures. The cells were stained with Lugol's solution to facilitate the counting procedure. Cells in a sample of the cultures were concentrated by centrifugation (600 × gravity) and the length and width of not less than 20 cells was measured using an ocular micrometer calibrated in an optical system of 1440 × magnification.

Preparatory to determination of cellular volume, a large number of clay models were constructed to scale in the various shapes of chilomonads observed. The volume of these was determined by immersion in a known volume of water in a calibrated graduated cylinder and noting the rise of fluid level. It was found that the formula for a prolate spheroid could be employed with slight modification. Thus the following formula was applied:

$$V = 1.47\ \pi\ A^2B,$$

where V = volume if A = ½ of the cell's width and B = ½ of the cell's length. The final volume of cellular material per ml. of culture was calculated by multiplying the number of cells per ml. by the average cell volume.

Respiration of cells in 3-day cultures was determined by the direct method of Warburg (18). Cultures used for studies contained 60,000

to 130,000 cells per ml. Variation in cell number had no apparent effect upon the degree of inhibition by cyanide. The amount of cellular substance present was determined by drying a concentrated cellular suspension to constant weight in a tared Pyrex glass weighing bottle. Correction was made for salts contained in the suspending medium. By comparison of optical density readings of cultures used in respiration tests with the optical densities of samples used for determination of dry weight, the dry weight of the cellular material in the culture could be estimated. This information was used in calculation of the $Q_{O_2}$ values ($\mu$l $O_2$ per mgm. dry weight per hour).

Oxygen consumption was measured for 1.5 ml samples of culture in the main compartment of standard Warburg flasks of approximately 5.0 ml capacity. Side arms contained 0.2 ml of 4 N $H_2SO_4$ and 0.1 ml of N KOH was placed in each center well. For tests containing cyanide, flasks were prepared in the same fashion, except that the cell suspensions contained $5 \times 10^{-5}$ or $10^{-4}$ M KCN for cells grown in AM-AC or APG medium, respectively; center wells contained a balanced KOH-KCN solution (11). Tests were run for at least one hour.

## Results

The results of growth studies are shown in Table I. It is apparent that greater total protoplasmic growth occurs in AM-AC than in APG medium. Although cellular division may occur more rapidly in APG medium, cells are two to three times as large in AM-AC. Cyanide inhibition of growth is greater in AM-AC than in APG both in regard to cell number and to total protoplasmic mass. The average cell volume of normal and "adapted" cells in AM-AC is affected only very slightly by cyanide.

On the average, cells growing in APG and not previously exposed to cyanide are 17 per cent smaller in $10^{-4}$ M KCN than they are in absence of cyanide. On the other hand the average "adapted" cell in $10^{-4}$ M KCN is larger than are "adpted" cells in the absence of cyanide.

It appears that resistance may have been gained to a slight degree by cells "adpted" in AM-AC, but a greater degree of resistance is seen in cells adapted in APG medium. The resistance is especially noticeable in comparing values of protoplasmic volume per unit of culture volume. The inhibition index (percentage inhibition in normal cells divided by percentage inhibition in "adapted" cells) in

TABLE 1

The effect of potassium cyanide upon growth of normal and cyanide adapted *Chilomonas paramecium* as influenced by the type of nutrient available. Medium for growth: AMAC, ammonium-acetate; APG, four parts of 1 per cent proteose peptone and glucose and one part of AMAC. Data are average of 20 tests for each condition. Inhibition index = percentage inhibition of normal cells divided by percentage inhibition of adapted cells. Standard deviation in parenthesis.

| Medium for Growth | | Normal Cells | | Percentage Inhibition | Adapted Cells | | Percentage Inhibition | Inhibition Index |
|---|---|---|---|---|---|---|---|---|
| | M. Conc. KCN | 0 | $5 \times 10^{-5}$ | | 0 | $5 \times 10^{-5}$ | | |
| | Cells/ml. | 96,500 (1,050) | 20,000 (1,000) | 79 | 64,500 (2,600) | 21,600 (500) | 67 | 1.18 |
| AMAC | Cell length (μ) | 25.4 (1.8) | 24.5 (1.4) | | 25.3 (1.3) | 24.4 (1.5) | | |
| | Cell width (μ) | 10.7 (0.4) | 10.8 (0.9) | | 10.9 (1.0) | 10.9 (1.5) | | |
| | Cell volume ($\mu^3$) | 1670 | 1640 | 2 | 1730 | 1670 | 3 | |
| | μl cells/ml. culture | 0.161 | 0.033 | 79 | 0.112 | 0.036 | 68 | 1.16 |
| | M Conc. KCN | 0 | $10^{-4}$ | | 0 | $10^{-4}$ | | |
| | Cells/ml. | 105,000 (2,000) | 63,000 (600) | 40 | 114,000 (1,100) | 62,000 (900) | 46 | 0.87 |
| APG | Cell length (μ) | 17.1 (1.0) | 17.8 (1.1) | | 17.3 (1.1) | 17.7 (1.3) | | |
| | Cell width (μ) | 8.41 (1.0) | 7.67 (0.8) | | 7.75 (0.9) | 8.15 (0.8) | | |
| | Cell volume ($\mu^3$) | 725 | 605 | 17 | 595 | 880 | inc | |
| | μl cells/ml. culture | 0.076 | 0.038 | 50 | 0.068 | 0.055 | 19 | 2.63 |

APG is 2.63, indicating that normal cells are more sensitive to cyanide inhibition than are adapted cells.

In Table II are presented the respiratory rates. Again, greater activity is seen in cells grown in AM-AC. However, cyanide is more

TABLE 2

The effect of potassium cyanide upon respiration of normal and cyanide-adapted *Chilomonas paramecium* as influenced by the type of nutrient available Medium for growth: AMAC, Ammonium-Acetate, APG, four parts of 1 percent proteose peptone and glucose and one part of AMAC. Data are average of 20 tests for each condition. Inhibition index = percentage inhibition of normal cells divided by percentage inhibition of adapted cells. Standard deviation in parentheses.

| Medium for Growth | M Conc. KCN | $Q_{O_2}$ | | Percentage Inhibition | | Inhibition Index |
|---|---|---|---|---|---|---|
| | | Normal | Adapted | Normal | Adapted | |
| AMAC | 0 | 74.2 (2.3) | 76.5 (1.2) | | | |
| | $5 \times 10^{-5}$ | 59.2 (2.6) | 62.5 (1.9) | 20 | 18 | 1.11 |
| APG | 0 | 55.3 (3.5) | 43.7 (3.4) | | | |
| | $10^{-4}$ | 31.3 (2.8) | 40.6 (3.3) | 41 | 7 | 5.9 |

inhibitory to cells grown in APG medium. There is little indication that adaptation occurs in AM-AC medium, while on the other hand there is a very striking difference in the inhibition of normal cells and those previously exposed to KCN in APG medium. Adaptation against respiratory and growth inhibition takes place when cells are growing in medium with an available organic nitrogen source.

## Discussion

Respiratory inhibition by cyanide is generally taken as indication of a functional cytochrome-cytochrome oxidase system. Various observations in this regard have been reviewed by Commoner (4). However, the classical cytochrome oxidase may not necessarily be functional in all protozoa showing cyanide sensitivity (12, 5). Thus, the exact designation of the final electron transfer system in Chilomonas might still be in question although cyanide sensitivity has been demonstrated in this paper and by Elrod (6) and cytochrome oxidase activity has been reported by Hutchens (8). Nevertheless, it seems not improper at this point to assume that a functional cytochrome system accounts for much of the oxygen consumption by this flagellate.

One may only speculate upon the system or systems which might be involved with the cyanide-resistant fraction of respiration and the interesting possibility that such a mechanism might be involved in the observed adaptation.

Cyanide caused stronger inhibition of growth of normal Chilomonas in the inorganic medium in this study. One might at first conclude that this would indicate reaction of the inhibitor with materials in the more complex (APG) medium. However, the reverse is true for respiration tests; normal cells were more inhibited in complex organic medium. It may very well be that different mechanisms are involved in growth and in respiration.

Not only is the difference in effect upon growth and respiration indicated, but there is evidence in this study that processes of growth itself must be regarded separately in view of cyanide effects and apparent adaptation. In medium in which little adaptation is apparent (AM-AC), cyanide inhibits growth within cultures of either normal or adapted cells with little change in cell size. Thus processes of protoplasmic assimilation and cell division are equally affected. However, in a medium (APG), in which some adaptation against growth inhibition occurs, normal cells in cyanide are smaller, while adapted cells in a test concentration of cyanide are larger than in its absence. Thus adaptation involves greater resistance of processes concerned with protoplasmic increase, but with a reduced ability of the cells to divide. The APG medium used here is very similar to the medium in which Tetrahymena has been cultured during cyanide adaptation (14). However, in the latter instance, adaptation occurs with increased resistance against inhibition both of protoplasmic increase and of cellular division. Therefore, while the adaptation may be dependent upon the presence of a complex organic medium for both species, the means by which the growth processes may be altered is different in the two forms and it would appear that Tetrahymena accomplishes the adjustment more efficiently.

Adaptation of respiratory processes also seems to depend upon the presence of complex organic nutrient. It should be noted that resistance gained against respiratory interference was much greater than that involving growth. This is true for Tetrahymena as well, but to a lesser degree.

Perhaps the nature of the "adapted" metabolism may be further indicated by consideration of the normal utilization of exogenous sub-

strates by Chilomonas. The flagellate normally utilizes ammonium nitrogen for its best growth (3, 13) and the naturally-occurring amino acids are not normally utilized in oxidative metabolism (7). Acetate may be oxidized, but glucose is not normally utilized for that process (7). Hutchens *et al.* (9) has shown that acetate serves as an energy source and is assimilated as well. However, ammonium salts are utilized only as a nitrogen source; nitrogen in that form is not oxidized to furnish energy.

While further investigations must be carried out in order to draw definite conclusions, it might be proposed that the resistance to cyanide in adapted Chilomonas is related to increased amino acid metabolism. Cyanide-sensitive respiration is generally concerned with carbohydrate utilization (4) and metabolic pathways involving acetate metabolism in Chilomonas are similar to those involved in final oxidation of products of anaerobic glycolysis (10). On the other hand, Commoner points to the probability that amino acid and fatty acid utilization, occurring at a slower rate, are involved in the so-called cyanide-resistant fraction of respiration. Bacteria which display a normal resistance to cyanide have lower respiratory rates (1, 2). It was noted in this study that adapted chilomonads show a lower respiratory activity than do cells not previously exposed to cyanide.

At this point it is not possible to state with assurance the exact means by which induced resistance to cyanide has been effected in Chilomonas. However, utilization of the clonal culture, slow increase in cyanide concentration and failure to observe evidence of sexual processes of reproduction in the culture populations would minimize the probability of selection or genetic change. In the instance of Tetrahymena (15) it is indicated that genetic change would be a highly improbable explanation for resistance gained by similar technique.

## Conclusions

1. *Chilomonas paramecium* has been cultured in ammonium-acetate medium and in medium with reduced ammonium-acetate and added proteose peptone and glucose in attempts to effect adaptation to potassium cyanide.

2. Adaptation occurs in medium containing proteose peptone and glucose; little adaptation occurs in ammonium-acetate medium.

3. Resistance gained against cyanide inhibition of growth is con-

cerned primarily with processes of protoplasmic increase, while cell division processes remain strongly inhibited in adapted cells.

4. Respiration in cyanide-adapted cells in absence of cyanide is less than that in normal cells, but resistance to cyanide is greater in adapted cells.

5. It is proposed that metabolism in adapted cells is concerned with increased utilization of organic nitrogen (amino acid) materials.

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# THE DEVELOPMENT OF THE PRIMITIVE STREAK AND EARLY CHICK EMBRYO IN RELATION TO LOW $O_2$ PRESSURE

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## INTRODUCTION

THE information presented in this paper is a part of a series of studies on the development and function of the primitive streak associated with the early phases of embryo genesis in the chick. These studies will offer an analysis of primitive-streak and early-rudiment activities using various techniques and methods of approach.

A determination of the $O_2$ and $CO_2$ limits within which morphogenetic and cellular activities occur provide evidence concerning the conditions under which physical or chemical factors play their respective roles in eliciting particular morphogenetic movements and differentiation. Such evidence will provide suggestive information relative to possible and probable biophysical and biochemical processes involved in the differentiation of particular embryonic rudiments and body regions.

The purpose of the study described in this paper pertains to the developing streak and the differentiation of early body-form in relation to low $O_2$ pressures, the total atmospheric pressure being equivalent to sea level pressure.

The exposure periods to the changed $O_2$ conditions cover the phase of development beginning with the initiation of incubation of the egg and on to the beginning of early tail development, i.e., to approximately stages 16 and 17 of Hamburger and Hamilton, 1951. The latter stages possess head, pharyngeal, trunk, and beginning tail areas.

## MATERIALS AND METHODS

The eggs of first year, single-comb, white Leghorn hens within the first week after laying were used in the experiments. The eggs were kept at a temperature of from 12°-18° C previous to incubation. Very

large eggs or rough surfaced eggs were discarded. Most of the evidence was obtained from eggs laid in the late autumn, winter and early spring months as these eggs appear to show less abnormalities when exposed to unfavorable atmospheric conditions than do late spring, summer, or early autumn eggs.

The procedures employed in exposing the eggs to lowered $O_2$ pressures were as follows: six eggs were placed within a steel pressure-chamber of 2700 $cm_3$ capacity. The chamber was previously warmed for a period of many hours at a temperature of 37.5°-38° C. Further treatment of the eggs followed two different methods.

1. Eggs exposed to pure $N_2$ during the early incubation period experienced the following treatment: a pre-purified $N_2$ previously warmed to a temperature of 37.5°-38° C was permitted to flow for three minutes through the warm chamber containing the eggs under an increased pressure of 1/5 atmosphere. The first exposure was made immediately after the eggs were placed in the chamber. The chamber was then placed in an incubator at 37.5°-38° C for 20 minutes, the pressure being maintained at 1/5 of an atmosphere above the normal atmospheric pressure. Following this 20 minute period, the pressure was reduced gradually to the normal atmospheric level by slightly opening a valve. A second similar perfusion of pure $N_2$ was made at 1/5 atmosphere of increased pressure. The chamber was then placed in the incubator at 37.5°-38° C for another 20 minutes, when the pressure was reduced gradually to the normal level again. This procedure was repeated two more times. The chamber, with the pressure within reduced to normal atmospheric pressure was then placed in the incubator for the desired period of time. This procedure permitted the air within the chamber to be replaced by pure $N_2$. Moreover, a considerable amount of the dissolved or contained $O_2$ within the eggs was presumably removed by the perfusion under pressure with subsequent reduction of pressure.

2. When eggs were exposed to conditions where various percentages of $O_2$ were present, they were treated in the following manner: A two-stage metering regulator equipped with a flow-meter, which indicated the actual rate of flow of the gas, was attached to the cylinder containing the specific gas employed. From the flow-meter a latex rubber tube, ¼ inch in diameter, conveyed the gas into an humidifying chamber placed within the forced-draft incubator used in these studies. From the humidifying chamber the gas was conveyed through

latex rubber tubing to a heating chamber made of glass placed immediately above the heater of the incubator. From the heating chamber the gas was conveyed through winding ¼ inch latex tubing three feet in length to the chamber containing the eggs. A small glass tube containing a thermometer was inserted in the latter tube to record the temperature of the gas just before its entrance into the exposure chamber. The gas entered the egg chamber from above and passed out of the chamber from the side about 1 inch from the bottom. A latex rubber tube conveyed the gas to the outside of the incubator where quantitative tests of the gas passing through the system were made by the use of a Scholander gas analyzer.

For the first 3 hours after the eggs were placed in the egg chamber the rate of flow was maintained at 0.6 liters per minute. This permitted the chamber and the eggs to be washed thoroughly with the particular gas used. Observations with the Scholander gas analyzer demonstrated that after 10-15 minutes of gas flow at 0.6 liters per minute, the gas at the outlet of the chamber approximated closely the percentages of the respective gases at the inlet to the chamber. For the remainder of the exposure period the rate of gas flow was reduced to 0.1 liter per minute. The number of hours of exposure was recorded as beginning at the end of the initial washing period.

In this study, 0.5% $CO_2$ was used with the various percentages of $O_2$. As shown in another study to be reported, the presence or absence of $CO_2$ effects marked changes in growth and development in relation to low $O_2$ presence especially in the definitive streak and later stages. That is, the effect of $CO_2$ appears to be more marked during the latter part of the first day and during the second and third days than during the early part of the first day.

Six eggs were used for each exposure with a corresponding 6 eggs as controls incubated in a forced-draft incubator. Each experiment was repeated six times, making a total of 36 experimental eggs and 36 control eggs for each exposure shown in Tables I and II. The blastoderms were observed in the living state and later as fixed, stained preparations. Formol-nitric solution followed by Smith's fluid was used in the fixation process for blastoderms during the first day of incubation, while Bouin's fluid was employed for older stages. Alum-cochineal was used in staining.

The gases were obtained from the Ohio Chemical and Surgical Equipment Company.

## Variations in the Developmental State of Unincubated Blastoderms

Any consideration of the effects produced by exposing eggs to changed environmental conditions should take into account the fact that the unincubated blastoderm of the chick embryo varies in its degree of development. A description of the variable development present in the unincubated and early incubated blastoderm will be set forth in another paper. For present purposes, it may be stated that the development of the blastoderm at the time of laying varies from that of a disc-shaped mass of cells without distinct pellucid and opaque areas to an older condition where rudimentary pellucid and opaque areas are present. In the latter state, the early phases of second layer or hypoblast formation are present. Most of the unincubated blastoderms exist in the older state of development, but some of the young and intermediate stages exist. Variations in response to exposure of low $O_2$ pressures therefore may be expected.

## Experimental Results

The results obtained following exposures to various low levels of $O_2$ is set forth in Tables I and II. In Table I the stages of development presented in column 2 represent the degrees of normal development reached at the various $O_2$ levels. In Table II the results of longer exposures are shown. The latter exposures produce deleterious effects but not to the degree shown in Table I, column 6.
Tables I and II here

## Summary and Discussion

1. It is evident from the data presented above that there are two aspects of differentiation to be considered in relation to the $O_2$ and $CO_2$ pressures used in these experiments, namely, the development and functioning of the primitive streak, and the realization and development of the various rudiments as the early embryonic body assumes form.

a. *Oxygen levels concerned with primitive streak development and function.* The initial phases of primitive streak development occur in the air-mixture containing 2% $O_2$, the broad or intermediate streak forms at a 4% $O_2$ level, and the early definitive or transitional streak in the mixture containing 6% $O_2$. A level of 6% $O_2$ also appears to be ample for the functioning of the definitive streak and head process

TABLE 1
Development of the early chick blastoderm at low $O_2$ levels

| Gas used | Stage of Development | Initial Time Exposed | Temperature | Development after removal to forced-draft incubator at 37.5 to 38°C for an additional 24 hrs. following initial exposure shown in column 3. | Development after further exposure to same conditions in exposure chamber beyond time shown in column 3. |
|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| Pre-purified $N_2$ | Pre-primitive streak blastoderm. | 5 hrs. | 37.5°C to 38°C | Development normal, but may show retardation. | Exposure from 8-12 hrs. produces general breakdown of blastoderm. |
| 2% $O_2$; 0.5 $CO_2$; remainder $N_2$ | Early streak Fig. 1. | 8-12 hrs. | 37.5°C to 38°C | Development normal | Exposure for 24 hrs. may produce elongated, degenerate streaks and/or degenerating blastoderms. Fig. 2. |
| 4% $O_2$; 0.5 $CO_2$; remainder $N_2$ | Broad streak Fig. 4. | 12-16 hrs. | 37.5°C to 38°C | Development normal, but may show higher death rate than controls. | Exposure for additional 24 hrs. produces degenerate blastoderms. In small number of cases attempts at early neurulation may occur. Figs. 7, 15, 19. |
| 6% $O_2$; 0.5 $CO_2$; remainder $N_2$ | Definitive, head process, & beginning head fold stages. Primitive groove & pit not deep. Figs. 3, 5, 6. | 20-24 hrs. | 37.5°C to 38°C | Development normal, but some embryos show retardation. | Marked degeneration of head structures when incubation continues into the second day; somite formation arrested after first 3 or 4 pairs. Figs. 8, 16. |
| 8% $O_2$; 0.5 CO ; remainder $N_2$ | About 4-5 somite stages; primitive streak normal. Fig. 10. | 24-28 hrs. | 37.5°C to 38°C | Development normal. | Degeneration of head structures when incubation continued into the late second day; somites arrested at about 7-9 pairs. Figs. 11, 18. |
| 10% $O_2$; 0.5 $CO_2$; remainder $N_2$ | About 12-15 pairs of somites. Figs. 9, 20. | 38-42 hrs. | 37.5°C to 38°C | Development normal, but some embryos may have eye defects. | Continuation of incubation to 48 hrs. generally results in eye defects in many embryos. Figs. 23, 24. |
| 12-14% $O_2$1 0.5 $CO_2$; remainder $N_2$ | Development normal but accelerated, especially at 14% $O_2$ level. | Into early 3rd day | 37.5°C to 38°C | Development normal, but slightly accelerated, especially at 14% $O_2$ level. | Development accelerated considerably when continued to end of 3rd day at 14% $O_2$ level. (Compare Figs. 21 & 22.) |

Drawings of blastoderms prepared as indicated under Materials and Methods
Reduced ½ on reproduction

Figure 1. Typical blastoderm exposed to 2% $O_2$, 0.5 $CO_2$ and remainder $N_2$ after exposure period of first 14 hrs. of incubation. Primitive streak in early stage.

Figure 2. More favorably preserved blastoderm exposed as in Figure 1 for first 24 hrs. Primitive streak elongated and somewhat indefinite. Blastoderm shows disintegrative tendencies.

Figure 3. Primitive streak and area pellucida of blastoderm exposed to 6% $O_2$ first 20 hrs. of incubation. The streak appears normal but primitive groove and primitive pit are shallow.

Figure 4. Blastoderm exposed to 4% $O_2$ for first 16 hrs. of incubation. Observe that area pellucida is round in shape. The streak is in the broad, fuzzy stage. If blastoderms are retained at this $O_2$ level for an additional 12 hrs. of incubation there is a tendency for the area pellucida to enlarge but also to retain its round shape.

Figure 5. Older embryo including primitive streak obtained from egg exposed to 6% $O_2$ for first 24 hrs. of incubation.

Figure 6. Head-process stage obtained from egg exposed to 6% $O_2$ for first 24 hrs. of incubation.

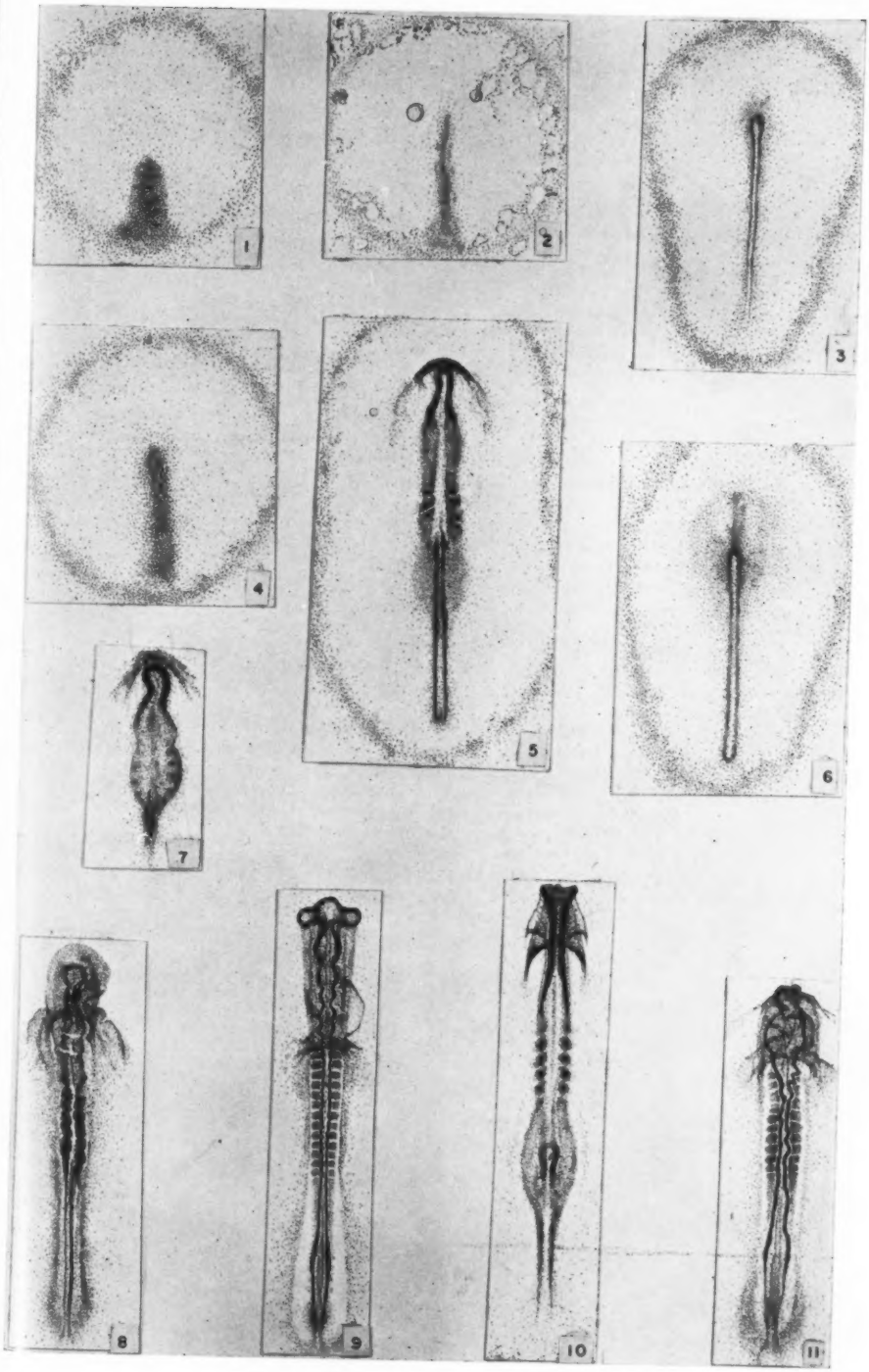
Figure 7. Abortive embryo obtained from egg exposed to 4% $O_2$ for first 42 hrs. of incubation. Compare with Figure 15. Exceptional blastoderm; not typical.

Figure 8. Abortive embryo exposed to 6% $O_2$ for 44 hrs. Observe that primitive streak appears to be functioning well but embryogenesis is suppressed. Compare with Figure 16.

Figure 9. Embryo exposed to 10% $O_2$ for 40 hrs. Compare with Figures 20, 23, 24.

Figure 10. Embryo from egg exposed to 8% $O_2$ for 28 hrs.

Figure 11. Embryo exposed to 8% $O_2$ for 44 hrs. This embryo is developmentally blocked. If eggs containing such embryos are removed to normal conditions in a forced-draft incubator they will continue to develop but the head will show abnormalities.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## PLATE II

Photomicrographs of early blastoderms and developing embryos. Specimens prepared as indicated under Materials and Methods. Reduced ½ on reproduction.

Figure 12. Normal primitive streak at end of 18 hrs. of incubation.

Figure 13. Broad or intermediate streak 14-16 hrs. of incubation at 4% $O_2$ level.

Figure 14. Broad streak, 4% $O_2$ level, after 24 hrs. of incubation. Observe evidence of blastodermic disintegration. See Figures 15 and 19.

Figure 15. Primitive streak and abortive embryogenesis after 43 hrs. of exposure at 4% $O_2$ level. This type of result is exceptional. As a rule, blastoderms begin to show evidence of degeneration, as shown in Figure 14, during the early second day of incubation when maintained at the 4% $O_2$ level. Compare with Figure 19.

Figure 16. Embryo at 43-44 hrs. of incubation at 6% $O_2$ level. The cephalic end is disturbed greatly but the trunk region is resisting the low $O_2$ effects. Primitive streak region appears quite normal. Compare with normal embryo Figure 17. This type of result is general at the 6% $O_2$ level. Compare with Figure 8.

Figure 17. Control embryo, 44 hrs. of incubation.

Figure 18. Typical result at 8% $O_2$ level, 44 hrs. of incubation. This embryo represents an almost completely blocked developmental condition with the exception of the primitive streak area.

Figure 19. Another exceptional 4% $O_2$ level embryo at 43 hrs. of incubation. Blastoderm undergoing degeneration. Developmentally blocked as of the early part of the second day's incubation. Compare with similarly blocked embryo, Figure 15.

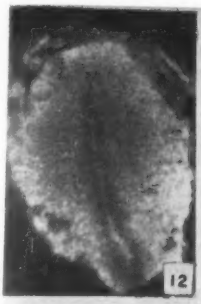
Figure 20. A 10% $O_2$ embryo at 40 hrs. of incubation, photographed ventral side up to show heart. Optic vesicles appear normal. Compare with Figures 23 and 24.

Figure 21. A 12% $O_2$ embryo at end of 3rd day of incubation.

Figure 22. A 14% $O_2$ embryo at end of 3rd day.

Figure 23. Ten percent $O_2$ at 43 hrs. of incubation. Observe optic vesicles are smaller than normal.

Figure 24. Ten percent $O_2$ embryo at 40 hrs. Optic vesicles irregular in size.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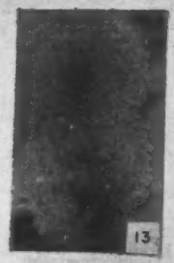
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TABLE 2

Development of early chick blastoderm after removal to forced-draft incubator following longer initial exposure than that shown in column 3, table 1, but not as long as column 6

| Gas used | Initial time exposed in chamber | Time exposed in forced-draft incubator following initial time in chamber | Results |
|---|---|---|---|
| 1 | 2 | 3 | 4 |
| Pre-purified $N_2$ | 5-8 hrs. | 36 hrs. | Embryos appear normal morphologically but may be reduced in size and retarded when compared with controls. |
| 2% $O_2$; 0.5 $CO_2$; remainder $N_2$ | 15-18 hrs. | 36 hrs. | Incidence of death higher; most embryos morphologically normal but retarded in development. |
| 4% $O_2$; 0.5 $CO_2$; remainder $N_2$ | 24 hrs. | 24 hrs. | Incidence of death higher; most embryos normal but retarded in development when compared to controls. |
| 6% $O_2$; 0.5 $CO_2$; remainder $N_2$ | 32 hrs | 24 hrs. | Retardation of embryos; head structures abnormal in 20-30% of embryos; some embryos normal. Trunk region normal in all embryos. |
| 8% $O_2$; 0.5 $CO_2$; remainder $N_2$ | 42 hrs. | 24 hrs. | Retardation of development; head structure abnormal in 60-65% of embryos; some embryos normal. Trunk region fairly normal in all embryos. |
| 10% $O_2$; 0.5 $CO_2$; remainder $N_2$ | 48 hrs. | 24 hrs. | Eye defects in about 30% of embryos; some embryos retarded; many normal. |
| 12-14% $O_2$; 0.5 $CO_2$; remainder $N_2$ | 72 hrs. | none | Embryos normal but larger than controls and accelerated in development. |

stages in which cellular migrations occur anteriorly from Hensen's node and laterally from the sides of the streak. However, although cell division and the segregation of cells from the streak area occur at the 6% $O_2$ level it is to be observed that deep invaginations in the primitive pit and primitive groove are absent. (See Table I, 6% $O_2$, column 2.) That is, involution of cells and their later slithering movement away from the streak appears to require a lower concentration of $O_2$

than definite infolding or invagination movements. This odd behavior on the part of the primitive streak is evident particularly at the 4% $O_2$ level. At this level of $O_2$, as indicated in Table I, 4% $O_2$, column 6, and in figures 7, 15, and 19, a considerable attempt on the part of the streak to function is evident even when the typical grooved form of the streak is suppressed. Exposures to mixtures of air containing only 8% $O_2$ demonstrates that at this level the streak functions well. However, one would not assert that the 8% $O_2$ level is most beneficial for the streak's functioning, for other, unpublished data, indicate that an increased pressure of from one to two added atmospheres of air accelerates streak-function from the transitional streak stage onward. The primitive streak therefore appears to able to carry out its functions under a wide range of $O_2$ levels.

Relative to the low level of $O_2$ required in the early stages of primitive streak development it is interesting to observe that Fraser, 1956, in a study of respiratory activities of the developing streak found that cytochrome oxidase, as exhibited by the Nadi reagent, did not appear until the intermediate-streak stage. At the definitive-streak stage Fraser found evidence of the oxidase along the streak and in the area of Hensen's node. In the head process stage, Moog, 1943, using a similar technique described the presence of cytochrome oxidase in the node area from which it spread disted along the streak and the embryonic axis. The most sensitive area, according to Moog, was the immediate locus of the node. Also, at the head process stage, the indophenol blue color clearly appeared in the node after about 4 minutes. A similar condition was present at 24 hours but at 28 hours or when the embryo possessed 5-10 pairs of somites the color reaction began to appear in 2-3 minutes with "the anterior end of the primitive streak and the neuropore" as "points of equally high intensity, from which the color spread posteriorly." At 50 hours or from 24-28 somite stages the color appeared "in the former primitive streak region" shortly after 2 minutes.

The findings of Fraser and Moog relative to the appearance of cytochrome oxidase closely parallels the observations in this paper, namely, a gradual need for $O_2$ as the streak develops, with the high point centered around the nodal area. Moreover, a high point of nodal activity appears to be reached gradually up to the 24-28 somite stages, for according to Moog's data, the time necessary for the indophenol blue color to appear drops from 4 minutes at the head process stage to 2 minutes at the 24-28 somite stages.

b. *Oxygen levels concerned with realization of the embryonic rudiments.* While the $O_2$ requirements for the realization of the primitive streak and its later functioning are relatively low, the need for $O_2$ rises as the emerging embryonic-rudiments begin to differentiate. See figures 7, 15, and 19. In this respect, two main regions of the emerging embryonic body are evident, namely, the head area which requires a higher level of $O_2$, and the remainder of the body which appears to be able to get along on a lower $O_2$ presence. As the anterior part of the embryo begins to emerge low levels of $O_2$ prove to be injurious to the developing anterior part of the forming neural tube. For example, when eggs are exposed to a level of 6% $O_2$ into the later hours of the second day drastic abnormalities in the head region appear in many embryos. (See 6%, Table I, column 6, also figures 18 and 16.) The developing trunk structures although retarded are more normal while the primitive streak appears normal. (Compare also Table II, 6% for the first 32 hours.) At 8% $O_2$ the head area may be drastically disturbed after exposures into the later hours of the second day. The trunk region, on the other hand, remains fairly normal in its development after such treatment. (Table I, column 6, Table II, column 4, and compare figs. 11, 17, and 48.) Even at 10% $O_2$ when the exposure is continued to the end of the second day of incubation the head shows marked sensitivity, particularly in the eye areas. (Table I, 10% column 6; Table II, 10% column 4. See also figs. 23 and 24.) The air mixture containing 12 to 14% $O_2$ is adequate through the first two days, and even into the third day, of development. (See Table I, 12-14% $O_2$ and Table II, 12-14% $O_2$, also figs. 21 and 22.)

The above reactions to the various mixtures containing low $O_2$ levels agree in some respects and disagree in others with the observations of Moog relative to the indophenol blue reaction (op. cit.). For example, Moog observed in embryos of 5-10 somites points of high color intensity of indophenol blue in the anterior neuropore at the tip of the forebrain, optic cups, otic vesicles, etc. She also observed that "within 3 or 4 minutes" the coloration spreads "downward through the head and upward along the trunk" p. 226. At 24-28 somites, color was observed "within 2 minutes in the heart and also in a region encompassing the anterior face of the forebrain, eye, and the nares, etc." Also "the color deepened quickly in the heart, and eye, and spread rapidly enough through the trunk, according to the usual gradients"

p. 227. The data acquired in the present study shows the necessity for relatively high $O_2$ usage in the head structures, and this observation fits well with the evidence for the presence of cytochrome oxidase in the same area indicated by Moog. However, in the trunk region, including the heart, a somewhat lower level of $O_2$ suffices, and this result does not compare favorably with the observations of Moog who noted a rapid development of a color gradient along the trunk. The observations in this present study suggests that a definite gradient along the trunk as observed by Moog does not exist; the line of demarcation with respect to low $O_2$ tolerance between the head and trunk regions is precipitous.

The real meaning of the indophenol blue reaction used to indicate the presence of indophenol (cytochrome) oxidase is not clear. Fraser, 1956, p. 80, found a "dark, blue-purple coloration on the Nadi reagent" on the opaque cell-area peripheral to the germ wall in pre-streak and early streak blastoderms but he found none in the pellucid area of the pre-streak blastoderm. Fraser ascribes this blue color as "due mainly to a non-enzymatic mechanism." This may indicate that there may be a factor or factors involved in the indophenol oxidase reaction other than that or those pertaining to metabolism involving the use of $O_2$.

Other workers have obtained results which indicate differences of $O_2$ usage by various areas of the developing chick blastoderm including different structures of the developing embryo. Hyman, 1927, using potassium cyanide, Rulon, 1935, and Miller, 1941, employing the Janus green reduction method, observed distinct regional differences closely paralleling the observations of Moog, 1941. On the other hand, Philips, 1941 and 1942, found an increase in $O_2$ consumption up to the definitive streak and head-process stages, but he concluded "that the level of the rate of oxygen consumption of the whole area pellucida and embryo remains relatively constant between the definitive streak stage and the seventh day of development" 1942, p. 96. He also disagrees with the interpretations of Rulon, 1935, and Miller, 1941, that the node-area of the head-process stage represents a locus of higher respiratory activity.

2. *Other work involving $O_2$ and the early chick blastoderm.* A concept held by some observers of chick development during the early part of the 19th century was that the hen's egg could develop independent of air. In 1834, Theodor Schwann by substituting hydrogen for air, came to the conclusion that air was essential and that the

primitive streak would not form without air. (See Needham, 1931, p. 617.) Wesselkin, 1913, used a method in which he substituted a part of the air by hydrogen. The changed mixture was passed through a receptacle containing the incubating eggs. According to his observation 5% $O_2$ permitted eggs to develop normally, although somewhat retarded, for the first 48 hours. Riddle, 1924, using a "washed gas of known oxygen (and carbon dioxide) concentration" which was passed through a sealed chamber by means of "hydrostatic pressure," reached the conclusion that "no dove or pigeon embryos enclosed in adequate shells of normally laid eggs have proved capable of living for as much as 24 hours in a moisture-saturated atmosphere analyzing less than 10.0 percent of oxygen." Relative to the chicken embryo he reached the conclusion that 11.4% $O_2$ was too low for the early stages of incubation up to 20 hours.

The data obtained in the present study substantiates the conclusions of Schwann but does not harmonize with the views of Wesselkin or Riddle.

3. *Retardation effects.* The results obtained in the present study suggest that a lagging or retardation of the normal rate of development is an initial deleterious response to exposures to low levels of $O_2$ in mixture with 0.5 $CO_2$ and $N_2$. This lagging effect produces embryos which develop more slowly and are slightly smaller. The retardation of development appears to precede the more malevolent, abortive attempts at rudiment differentiation. Therefore, the retardation effect may be regarded as developmental suppression, but one which is not severe enough to bring about definite abnormalities of organ differentiation.

Retardation in development is evident under 4% $O_2$ conditions following the broad streak stage, at 6% it begins to appear at the definitive streak and head-process stages, while at 8% $O_2$ retardation appears in the differentiating embryo following the early somite stages. However, at the 8% $O_2$ level, the primitive streak functions well, at least to the early tail bud stage. (See Table I, column 5; Table II, column 4.)

Developmental behavior at low oxygen levels may be summarized as follows: at a specific level of $O_2$, development progresses in a normal manner to a particular stage, depending upon the level of $O_2$ present. This period of normal development is then followed by a period of slower, retarded development, to be succeeded next by an abortive

attempt at development, and then finally by a complete blocking of development. If this blocking is not quickly relieved, disintegration and death follow. It is to be observed that primitive streak function is less easily blocked by lower $O_2$ levels than that of organ individuation and differentiation particularly in those organ rudiments in the cephalic end.

4. *Specific effects upon the central nervous system.* The data obtained in this study suggests that two general areas of response occur in the developing nerve tube. The hindbrain and the spinal cord are not readily affected by low $O_2$ levels whereas the mesencephalon and regions cephalic thereto are altered readily. The optic vesicles appear most sensitive to the low $O_2$ levels. See figs. 11, 16, 18, 23 and 24. Grabowski, 1957, also concludes that there are two main regions of the central nerve system of the chick which behave differently in development, namely, a posterior portion composed of the spinal cord and a posterior hindbrain and an anterior region beginning at about the level of the otic vesicles and continuing forward.

## Conclusion

1. The early pre-streak blastoderm of the chick can tolerate atmospheric conditions bordering upon complete absence of $O_2$ for the first 5-7 hours of incubation.

2. Primitive streak development requires $O_2$ in increasing amounts, although the initial stages of primitive streak development may occur at very low $O_2$ levels. The beginning streak requires at least 2% $O_2$, the broad streak 4% $O_2$ the definitive streak and head-process streaks 6% $O_2$. The definitive streak and later streak stages are more elongate and deeply grooved (invaginated) at 8% $O_2$ than at 6% $O_2$. Six percent $O_2$ appears to be adequate for primitive streak function at least into the second day, but the individuation of the various rudiments, particularly of the head, is disturbed.

3. The primitive streak is able to function through a wide range of $O_2$ levels. The lowest level is slightly above 4% $O_2$ whereas the higher level is considerably above the normal 21% $O_2$ level present at sea level. (See also Nelsen 1955.)

4. The involution of cells from the surface epiblast to form the streak's substance and the slithering of cells away from the streak area once they have been involuted require less $O_2$ than do the invaginative movements necessary to form a deep primitive groove and deep primitive pit.

5. Six percent $O_2$ appears adequate without the induction of abnormalities in the head structures through the head-process stage and possibly to the early head-fold and beginning somite stages.

6. Eight percent $O_2$ does not produce abnormalities when the blastoderm is exposed to it through the first somite stages, possibly as far as 5-7 pairs of somites.

7. Ten percent $O_2$ is adequate up toward the end of the second day of incubation or to about 10-15 pairs of somites, although abnormalities in the developing eye may appear with this exposure.

8. Twelve to 14% $O_2$ permits normal development of all features during the first two days of incubation or through stages 14 and 15 of Hamburger and Hamilton, 1951. The 0.5% $CO_2$ level with the 14% $O_2$ appears to accelerate development slightly.

9. Two levels of minimal $O_2$ requirement are evident, namely, the level necessary for the development and function of the primitive streak and the level required for the normal development of the individuating rudiments. The latter phenomena demand a higher $O_2$ level than the former. The head structures particularly are sensitive to lowered levels of $O_2$.

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